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ICHTHYOCRINOTOXICITY OF MARINE CATFISHES OF MUMBAI COAST

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN MARICULTURE

OF THE

CENTRAL INSTITUTE OF FISHERIES EDUCATION

(DEEMED UNIVERSITY)

MUMBAI – 400 061, INDIA

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JULY, 2000

Dedicated to the Memory of
my Beloved Father

CERTIFICATE

Certified that the thesis entitled **Ichthyocrinotoxicity of Marine Catfishes of Mumbai Coast** is a record of independent *bona fide* research work done by **Mr. Ashutosh Dharmendra Deo** during the period of study from November 1995 to July 2000 under our supervision and guidance for the degree of **Doctor of Philosophy in Mariculture** and that the thesis has not previously formed the basis for the award to the candidate of any Degree, Diploma, Associateship, Fellowship or any other similar title.

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DECLARATION

I do hereby declare that the thesis entitled **ICHTHYOCRINOTOXICITY OF MARINE CATFISHES OF MUMBAI COAST** is a record of *bona fide* research work done by me during the tenure of my study period of November, 1995 to July 2000 and that the thesis has not previously formed the basis for the award to me of any Degree, Diploma, Associateship, Fellowship or other similar title of any University.

Ashutosh D. Deo

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ABSTRACT

Epidermal secretions of two marine catfishes *Arius dussumieri* and *Osteogeneiosus militaris* from Mumbai waters were studied for their biotoxic properties. Crude mucus extract obtained by scrapping was lyophilized for use as crude toxin. This crude toxin was further fractionated using DEAE Cellulose column chromatography. Bioassays involving Kausauli strain male albino mice of 20±2 g weight revealed lethal activity in crude mucus extract of both the species, with MLD values of 0.25 ml (*A. dussumieri*) and 0.50 ml (*O. militaris*) per mouse. 24 Hr LD_{50} values were calculated to be 20.06 mg/kg and 25.86 mg/kg for *A. dussumieri* and *O. militaris* respectively.

Potent hemolytic activity upto 16 HU (*A. dussumieri*) and upto 8 HU (*O. militaris*) were exhibited while hemagglutnation was only partial. Crude extracts also exhibited edematic activity, upto 154% ER in *A. dussumieri* and 137% ER in *O. militaris*. Partially purified toxin of both the fish had pronounced hemagglutination activity of 32 HAU. In case of the partially purified fractions, 2 lethal factors were discernible in each species. All lethal factors had a parallel hemolytic or edematic activity. In addition to these, fractions of both fishes each had 3 hemolysins and 2 edema factors. Avil ® and Dolonex ® blocked the edematic acivity, the latter being more effective than the former; Atropine ®, on the other hand, enhanced the edematic activity.

These toxic fractions were further separated on HPLC that indicated a compound resembling Cytochrome C to be the toxic factor. SDS-PAGE revealed the presence of at least 18 proteins in *A. dussumieri* and 16 proteins in *O. militaris*

crude mucus, with their molecular weights ranging between 10 and 100 kD. Gas Chromatography of the lipid fractions indicated predominance of Palmitic acid, Oleic acid, Stearic acid, Palmitoleic acid, Myristic acid, Lauric acid and Linoleic acid, a precursor of prostaglandins.

The crude mucus extract of both species exhibited wound healing activities in a CAM model with marked angiogenic activity. Area of wound decreased by 22.5% and 30.0% while hydroxyproline increased by 24.93% (*A. dussumieri*) and 25.52 % (*O. militaris*) and lysyloxidase by 9.09% (*A. dussumieri*) and 9.17% (*O. militaris*) in 7 days when 20.0 µl of crude extract was applied topically to each of the punched wounds in guinea pig.

Envenomated mice, upon autopsy revealed gross anatomical changes in the liver and lungs. Histopathological investigations revealed distended sinusoids, pycnotic and karyorrhectic nuclei and karyolysis in liver, and thickening of alveolar walls in the lungs as also blood clots in both the tissues. Damage to kidney and heart was negligible. Histology revealed, in both the species, specialized glands to be absent in the skin while a mucus aggregation around, and glycoprotein-like substances, inside the lumen of the spines and might have a role in the toxicity.

Toxicity was not correlated with food of the fish but with season and the Condition Factor (K_n) of the fish indicating stress do the causative factor for increased epidermal secretions. Possibilities exist that Stress Proteins (SP) (=Heat Shock Proteins, HSP) 68 and 70 might be involved in the toxicity, wound healing and other bioactivities. A primary role of defence can be attributed to the mucus and the lethality could be a synergistic effect of the different bioactivities.

सारांश

प्रस्तुत शोध में दो समुद्री कैट फिश एरियस.डुसुमेरी एवं ऑस्ट्रियोजिनिओसस.मिलिटेरिस के त्वचीय स्त्रवण के विषाक्तता का अध्ययन है । प्रयोग में लाए गए मछलियों की औसत आकार 26.5 से.मी./220 ग्राम एवं 24.70 से.मी./180 ग्राम, ए.डुसेमेरी एवं ओ.मिलिटेरिस के लिए क्रमशः थी । जिससे 1.0 एवं 0.8 मि.ली. अपरिष्कृत सार प्राप्त हुआ जिसे लायोफिलाइज करने पर 40 एवं 32 मि.ग्रा. पाउडर प्राप्त हुए । इस अपरिष्कृत विष को डी.ई.ए.इ. सेल्यूलोज कॉलम पर परिष्कृत किया गया और उसका प्रभाव चूहों पर आई.पी. सूई लगाकर किया गया । औसत मृत्यु खुराक 0.25 मि.ली. एवं 0.50 मि.ली. क्रमशः ए.डूसूमेरी एवं ओ.मिलीटेरिस के लिए पाया गया । रक्त विखण्डन की क्षमता अधिकतम 16 एच.यू. एवं 8 एच.यू. दोनो मछलियों के अपरिष्कृत श्लेष्मा में पायी गयी । रक्त संचयन की प्रक्रिया अपरिष्कृत श्लेष्मा में नहीं के बराबर थी जो अर्ध परिष्कृत करने पर काफी प्रभावशाली पाई गई ।

अर्ध परिष्कृत श्लेष्मा में दो घातक तत्व मौजूद थे जो या तो रक्त विखंडक या रक्त शोफ कारक तत्व के साथ थे । इन विषों को एच.पी.एल.सी. द्वारा अलग किया गया जो साइटोक्रोम सी के आस पास निसृत होता हुआ पाया गया । इनेवट्रोफोरोसिस द्वारा दोनों मछलियों में क्रमशः 18 एवं 16 प्रोटीन बंध पाए गए जो 10 के.डी. से 100 के.डी. तक वितरित थे । गैस क्रोमोटोग्राफी द्वारा किए गए वसीय अम्ल अध्ययन से पता चला कि पामीटिक, ओलिक, स्टेरिक, पालमीटोलिक, माइरिस्टिक, लारिक अम्ल के साथ साथ लिनोलिक अम्ल भी मौजूद था जो पोस्टाग्लैंडिन के निर्माण में मदद करता है ।

दोनों मछलियों के अपरिष्कृत श्लेष्मा में घाव भरने की क्षमता भी पाई गई । कैम मॉडल में भी नलिका निर्माण की प्रक्रिया काफी हद तक पाई गई । घाव के क्षेत्रफल में 22.5 एवं 30.00 प्रतिशत की कमी पाई गई । हाइड्रोक्सी प्रोलीन का लेवल 24.93% एवं 25.52% तथा लाइसिस आक्सीडेज 9.09 एवं 9.17% सात दिनों में 20 माइक्रोलीटर प्रति घाव अपरिष्कृत श्लेष्मा लगाने से प्राप्त हुई ।

विष के प्रभाव से मरे हुए चूहों के ऊतकीय अध्ययन से पता चला कि यह लीवर एवं फेफड़े को काफी नुकसान पहुंचाता है । जबकि हृदय और वृक्क काफी हद तक सुरक्षित पाए गए ।

त्वचा के ऊतकीय अध्ययन से पता चला कि इसमें कोई भी ग्रंथी नहीं है जबकि स्पाइन के भीतक ग्लादईको प्रोटीन जैसा पदार्थ वलयाकार अवस्था में मिला ।

विष का मछलियों के भोजन से कोई सम्बन्ध नहीं पाया गया जबकि कंडीशन फैक्टर के साथ यह संबंधित था । हीट शॉक प्रोटीन के होने की संभावना भी प्राप्त हुई । ऐसा अनुमान लगाया गया कि विष का यह प्रभाव विभिन्न गुणों का मिश्रित प्रभाव है ।

CONTENTS

CHAPTER 1 : GENERAL INTRODUCTION

1.1: Ocean, the Last Frontier

Artists and poets, sailors and explorers, merchants and treasure hunters, since the dawn of civilization all have down to the water's edge, to gaze in wonder at the majesty of the sea, in all its vicissitudes, its relentless and unpredictable moods and the myriad life forms above and below its restless surface.

The oceans constitute the major units of environment and covers and extensive area of 361 x 10^6 km^2, i.e. about 70% of earth's surface to an average depth of 3800 m, bringing land and water ratio to 1:2.43. The continental shelves (0-200 m deep) cover approximately 5% of the earth's surface, slopes (200-300 m) cover 13%, abyssal depths of 3000-6000 m cover 51%, and hadal depths more than 6000 m cover less than 2%. The total volume of the oceans is 1.368 x 10^9 km^3, providing living space that Cohen (1994) estimated to be 168 times that offered by terrestrial habitats. This immense volume of seawater has an overwhelming influence on man. As a biological environment it harbors 4/5^{th} of the earth's animal life representing over 500,000 species in 30 phyla (Marderosian, 1968).

The ocean and its manifold living and non-living resources have long attracted the close attention of mankind. Fleets have delved into oceanic expanses for thousands of years. It plays a central role in human life as a source of protein rich cheap food, activity of recreation, communications, scientific discovery, industrial exploitation, agricultural development, military advances and the recovery of historical artifacts and treasures.

The ocean exercises an increasingly perceptible influence on the life of each nation and on the entire system on the community of nations and their politics. The ocean is becoming a major source of oil, natural gas, iron, manganese, etc. The solutions for global problems of energy, food, and other primary materials depend upon the level of mastering the sea and it has always been regarded as a great potential source. The variety of form and function found in the ocean represents a wealth of biological diversity. In fact the oceans are a major resource of mankind, which are yet to be explored and exploited to their full potential since the technology for ocean resource exploitation is basically in a state of infancy.

The interaction between man and the sea has, till date, projected the former as being the intrepid, insatiable and lascivious beneficiary, although the latter is impregnable. But this attitude of man seems to be the only feasible solution among the multifarious alternatives available for varied resources. The users of the oceans are no longer just a handful of maritime powers but the world community as a whole. Every country, whether developed or developing, coastal or landlocked, has a stake in the ocean today.

1.2: Marine Fishery Resources of India

Surrounded by sea on three sides of the mainland, India has a vast potential in terms of marine living and non-living resources. For ages India has been exploiting her seas, particularly by fishing which has since been the traditional occupation of the country's coastal fishermen community. Export of marine products is also not new to this country. Exports of pearl to countries such as through the Gulf to the ancient Roman Empire are well documented in

Indian history. However, fishing as an industry did not progress enough to contribute the national wealth till the nineteen fifties. The search of cheap protein and the advent of independence have paved the way for progressive onward march of this occupation, resulting in increased marine fish landings and subsequently in more export earnings. The declaration of the EEZ in 1977 has added a fillip for introducing larger vessels for exploiting marine fishery resources. Land areas being limited, their yielding capacity may not be able to be increased on par with growing food demands of millions over the globe. Hence, all the maritime countries pay more and more attention to the vast seas to meet the demands and India is no exception.

India is endowed with a long coastline of 8,129 km, 0.5 million sq. km of continental shelf, 2.02 million sq. km of EEZ (Anon, 1991). Region-wise, the EEZ off the west coast forms about 42.6%, off east coast 27.8% and around Andaman and Nicobar Islands about 29.6% of the EEZ. Utilizing the data on primary and secondary production, characteristics of the exploited stocks in the 0 to 50 m depth zone and the results of the exploratory surveys beyond 50 m depth, the Working Group constituted by the Government of India (Anon. 1991) assessed the cacheable annual marine fisheries potential to be 3.9 million tons, *viz.* 2.21 million tons from 0 to 50 m depth zone and 1.69 million tons from beyond 50 m depth in the EEZ. Resources wise, pelagic resources constitute 1.916 million tons, demersal resources 1.689 million tons and oceanic resources 0.295 million tons (Anon. 1991). On the basis of marine fisheries statistics collected from all along the Indian coast, the marine fish production during 1997 was estimated as 2.69 million tons (Anon. 1998).

Arius tenuispinis, A. thalassinus, A. dussumieri and *A. serratus* of the family Ariidae and *Osteogeneiosus militaris*.

In the northwest sector of Gujarat and Maharashtra, catfishes contributed 40% of the total catfish catch of the country, among which 80% was contributed by only four species, *A. dussumieri, O. militaris, A. thalassinus* and A. *tenuispinis* (Menon *et al.* 1992). They also estimated the contribution of *A. dussumieri* and *O. militaris* in Maharashtra to be 25% and 21.9% respectively, based on the average taken from 1979 -1988.

1.4: Biodynamic Substances from Marine Fishes

As man attempts to harness ocean resources, toxic marine organisms become increasingly important since they are an integral part of biological economy of the sea. More than 1,000 species are reported to be poisonous or venomous out of 500,000 species of 30 phyla, which the sea harbors (Russell, 1984). They are not only a health hazard but also represent an enormous resource for the discovery of potential therapeutic agents and will ultimately yield as many pharmaceutical leads as have been obtained from the terrestrial biosphere (Baker and Williamson, 1986).

A toxin is a substance possessing a specific functional group arranged in the molecules and showing strong physiological activity (Hashimoto, 1979). Biotoxins represent a somewhat more restrictive category, partly obscuring the fact that many "toxins" have the potential to be applied as a drug or pharmacological reagent, particularly after careful extraction, isolation and characterization (Baslow, 1971). Furthermore, even if direct use as a drug is not feasible because of potent or harmful side effects, the toxin can serve as a

model for synthesis or improvement of other drugs (Colwell, 1984). Many attempts are being made to develop useful drugs from the sea by screening for ant carcinogenic, antibiotic, growth-promoting (or inhibiting), hemolytic, hemagglutinating, analgesic, antispasmodic, hypotensive and hypertensive agents (Hashimoto, 1979) and even anti- HIV agents (Renn, 1993). Over 6,500 marine natural products have been isolated from marine organisms (Bhakuni, 1998).

1. 5: Classification of Biotoxins

Biotoxic organisms are generally classified into two types: i. Poisonous or Phenerotoxic organisms and ii. Venomous or Acanthotoxic organisms (Halstead, 1964).

1.5.1: Poisonous (Phenerotoxic) Fishes

These are organisms which are toxic only when they are consumed and can be further subdivided on the basis of the tissue in which the toxin is present, *viz.* Icthyosarcotoxic (flesh, musculature, viscera, or skin), icthyootoxic (eggs or roe, mature gonad) or icthyhaemotoxic (blood or serum).

1.5.1.a: Icthyosarcotoxic Fishes: In ichthyosarcotoxic fishes, the toxin can be either endogenous (eg: tetrodotoxin, ciguatera, etc). or exogenous (Concon, 1988). Since ichthyosarcotoxic fishes are generally identified with the kind of fish involved, these can be further sub grouped into the following subheads:

i. Ciguatera: Ciguatera is a circumtropical disease characterized by a wide array of gastrointestinal and neurological signs and symptoms (Vernoux and Lewis, 1997). Ciguatoxins arise from certain strains of the epiphytic,

benthic dinoflagellate *Gambierdiscus toxicus*, a common component of macro algal biodetritus throughout the warm waters (Lewis and Holmes, 1993). It stems from eating fish, captured mostly in inshore fisheries associated with coral reefs that have accumulated polyether sodium channel toxins known as Ciguatoxins (CTX) (Concon, 1988). Ciguatera is widespread in the Pacific Ocean (Gillespie, 1986), in the Indian Ocean (Quod *et al.* 1995) and the Caribbean Sea (Tosteson *et al.* 1988). More than 400 species of fish have been reported to be Ciguatoxic at one time or another (Halstead, 1994). Most of them are commercially important, eg: snappers, groupers, mullets, sturgeons, mackerel, barracudas, etc.

ii. Tetrodotoxic Fishes: Tetrodotoxin (TTX) was first isolated from a puffer fish (Tsuda and Kawamura, 1952) and then also from other cold blooded animals: the California newt (*Traicha torosa*), a goby (*Gobius criniger*), frogs (*Atelopus* species), Octopus (*Octopus maculosus*), a gastropod (*Babylonia japonica*), a starfish (*Astropecten polyacauthius*), and even Xanthid crabs (Kodama *et al.* 1985). Tetrodotoxin is one of the most potent marine toxins and causes many fatalities (Halstead, 1994). The origin of TTX and TTX-like compounds in nature remains uncertain. A bacterial origin for tetrodotoxin has been proposed to account for its presence in marine organisms including puffer fish (Yasumoto *et al.* 1986). Many bacteria, *Pseudomonas* sp. *Vibrio alginolyticus, V. fisheri, Alteromonas* sp. etc. have been suspected as causative agents (Okada and Niwa, 1998) but Matsumura (1998) claimed that TTX has an endogenous origin.

Tetrodotoxic fishes have worldwide distribution in both tropical and subtropical waters and found between the latitudes of 47^0 N and 47^0 S. Puffer

fishes show variability in the toxicity depending on the geography, season and sex (Concon, 1988). Female puffers are more toxic than the males, since ovaries tend to be more poisonous than the testes (Halstead 1994). No cooking or drying procedure destroys the poison.

iii. Gempylotoxic Fishes: The gempylids or snake mackerels are predatory oceanic or neritic pelagic fish, distributed in the Indo-Pacific, South American Pacific and tropical Atlantic Oceans and along the South African coastline. Gempylid fishes contain oil in their flesh and bones that is reported to be highly purgative (Concon, 1988). Diarrhea following ingestion of such fishes occurs rapidly, but without pain or cramps.

iv. Chimaerotoxic Fishes: The flesh, especially the viscera, of chimaeras is reported to be toxic. Halstead (1967) reported two species of ratfishes or chimaeras, *Chimaera monstrosa* and *Hydrolagus colliei* to be toxic. The chemical nature of toxin, its mode of action and the symptomatology of chimaera poisoning in humans is still unknown.

v. Cyclostomotoxic Fishes: The Cyclostomotoxic fish consists of four species of lampreys and one species of hagfish. The species of lampreys reported to be toxic include the Caspian lamprey, *Caspimyzon wagneri*, the river lampreys, *Lampetra fluviatilis* and *L. planeri* and a sea lamprey, *Petromyzon marinus*. The Atlantic hagfish, *Myxine glutinosa* of the family Myxinidae is reported to be toxic (Halstead, 1980). The slime, skin and flesh of cyclostomes are reported to be toxic. Usually the symptoms are mainly gastro intestinal effects, nausea, vomiting, tenesmus, and abdominal pain. The victim recovers after several days (Halstead, 1967). Exact chemical nature of the toxin is unknown.

vi. Elasmobranch Poisoning: Consumption of livers and flesh of some tropical sharks is the most common cause of this type of poisoning (Russell, 1965). The symptoms start 30 minutes after ingestion and include nausea, vomiting, diarrhea, abdominal pain, headache, joint aches, burning sensation in digestive system, and finally nervous breakdown. Death may occur in severe poisoning (Halstead, 1994).

vii. Clupeotoxic Fishes: These are members of the order Clupeiformes and include some of the herrings, anchovies, and related species. Apparently clupeotoxic fish become poisonous after eating certain planktonic organisms, such as toxic dinoflagellates. This intoxication is rare and resembles ciguatera but is very rapid acting and has a high mortality rate (Halstead, 1967). The first sign of Clupeotoxic fish poisoning in human is the sensation of sharp metallic taste in the mouth. Then follow signs of gastrointestinal disturbances, chills, clammy skin, hypotension, respiratory distress, coma, and finally death within 15 minutes. The nature of the toxin in Clupeotoxic fish is still unknown.

viii. Scombrotoxic Fishes: These fishes belong to the family Scombridae, the tuna related species. This type of poisoning occurs throughout the world, wherever scombroid fish are eaten. Besides scombroid fishes, it is also reported from non - scombroid fishes such as mahi-mahi, anchovies, etc. (Murray *et al.* 1982). The major causative agent of scombroid poisoning is histamine that forms in fishes due to bacterial action when they are improperly preserved. Histamine poisoned victim feels a sharp peppery taste in mouth immediately after consumption of toxic scromboid fishes. Symptoms consist of intense headaches, dizziness, throbbing of the carotid and temporal vessel,

epigastric pain, burning sensation in throat, cardiac palpitation, rapid and weak pulse, dryness of the mouth, nausea, vomiting, diarrhea, pruritis and respiratory distress. There is a danger of shock, and deaths have been reported (Halstead, 1994). However these symptoms last only 8-12 hours (Taylor, 1986).

ix. Ichthyoallyeinotoxic Fishes: Ingestion of certain type of reef fishes (mullet, goat fish) known to occur in the tropical Pacific and Indian Oceans can cause ichthyoalleinotoxic or hallucinogenic fish poisoning. Biotoxication may result from eating either head or flesh of the fish. The source and chemical nature of the poison is unknown (Concon, 1988). The poison affects the Central Nervous System and symptoms consist of dizziness, loss of equilibrium, lack of motor coordination, hallucinations, and mental depression. No fatalities have been reported (Halstead, 1980).

x. Ichthyohepatotoxic Fishes: This type of toxicity is mostly reported from Japan, where livers of certain edible species of fish *viz.* tunas, mackerel, sea bass, grouper, etc. are sometimes found to be toxic to eat. Nothing is known about the chemical nature of poisons involved, but it is believed that the intoxication is the result of hypervitaminosis A (Halstead, 1994). Symptoms of ichthyoheptotoxism consist of nausea, vomiting, fever, headache, diarrhea, joint pain, cardiac palpitation, and hepatic dysfunction; large areas of skin may peel off around the nose, mouth and head (Concon, 1988).

1.5.1.b: Ichthyootoxic Fishes: In these fishes, the toxin is generally restricted to the gonad or roe and the musculature and other parts of body are safe to eat. Most of the intoxication resulting from the ingestion of

ichlthyootoxic fish occurs during the reproductive season, during which the gonadal activity of the fish is at its peak. Examples include sturgeons, gars, pike, and minnows. Generally cooking is said to destroy most ichthyootoxins, but it cannot be relied upon as a completely safe procedure since the poison in some fish appears to be resistant to heat (Halstead, 1994).

1.5.1.c: Ichthyohemotoxic Fishes: These fishes have poison restricted in their blood or serum and are members of the order Anguilliformes, families Anguillidae, Congridae, Muraenidae and Ophichthyidae. eg: *Anguilla,* as also conger, moray and snake eels, etc. These eels are regarded as generally edible, except for the toxic blood. The exact nature of the toxin is unknown but the toxin from the eels, *Anguilla vulgaris, Muraena helena* and *Conger vulgaris* are reported to be proteinaceous (Russell, 1965). Cooking is said to destroy the toxic properties of eel blood (Halstead, 1994).

1.5.2: Venomous (Acanthotoxic) Fishes

Acanthotoxic or venomous fish produce their poison by means of glandular structures and are equipped with a traumatogenic device (teeth, spine, sting etc.) to purvey their venom (Halstead and Vinci, 1988). Most of the acanthotoxic fishes are bottom dwellers and non migratory and toxins secreted by them are readily destroyed by heat or gastric juices (Halstead and Vinci, 1988). Well-known examples are the rays, sharks, skates, catfishes, weaver fishes, scorpion fishes, etc. Symptoms include hypotension, respiratory distress, cardiac disorder, etc. (Hashimoto, 1979). Catfish spines are known to inflict painful wounds, which are often accompanied by edema, numbness, lymphadenopathy, paralysis and even gangrene (Russell and Brodie, 1974).

1.5.3: Ichthyocrinotoxic Fishes

Intermediate to the poisonous and venomous fishes are those that are categorized as crinotoxic fishes (Halstead, 1970); these have specialized secretory cells or glands in their skin but lack a parenteral mechanism. Glandular secretion is normally released in surrounding medium ie. water. Crinotoxins are often called mucus toxins. The poison glands of ichthyocrinotoxic fish assist in the defensive mechanism of the fish as warning or repellent substances (Halstead and & Vinci, 1988). Representative fish include certain filefish, puffer fish, trunkfish, boxfish, toadfish, goby fish, catfish etc. Symptoms of ichthyocrinotoxism are gastrointestinal distress, weakness, dermatitis, etc. (Hashimoto, 1979). Most of the victims recover within 24 - 36 hours.

1.6: Catfish and Toxicity

Catfishes are such fishes that exhibit both crinotoxic and acanthotoxic nature (Cameron and Endean, 1973). Envenomation from catfish is generally incurred as a result of handling the fish. When catfishes are agitated the dorsal and pectoral fin spines are firmly locked into a rigid extended position. A number of catfishes have prominent venom glands elaborated in their dorsal and pectoral stings and contact with these strings results in painful wounds (Halstead, 1970). Secondary infection following catfish sting envenomation is very common. Deformities in the hand or finger of fishermen are a common example of envenomation (Maretic, 1988).

Cameron and Endean (1973) described six species of catfish that produce crinotoxins. They also assumed that many catfishes other than the six

species are probably crinotoxic. Recently definite evidence that catfishes produce crinotoxins was obtained with two species, the Arabian Gulf catfish *Arius thalassinus* (Al-Hassan *et al.* 1982, 1985, 1986; Thomson *et al.* 1989) and the oriental catfish *Plotosus lineatus* (Shiomi *et al.* 1986, 1988). Both catfishes possess a proteinaceous toxin in the skin secretion, together with gland venom.

1.7: Scope of the Present Study

While comprehensive information of the fishery biology and resource potential of most of the marine catfishes of Indian water is available, knowledge on aspects of their crinotoxicity is scanty. The present study on two marine catfishes, *Arius dussumieri* and *Osteogeneiosus militaris*, that are abundantly available in Mumbai waters was taken up to obtain information on the nature of their crinotoxicity, its seasonal and size–wise variations if any, and correlation with general well being (condition factor) and feeding behavior.

This study also aimed at obtaining some valuable information on extraction of bioactive compounds for biomedical uses mainly on wound healing, which has been observed by Al-Hassan *et al.* (1983)

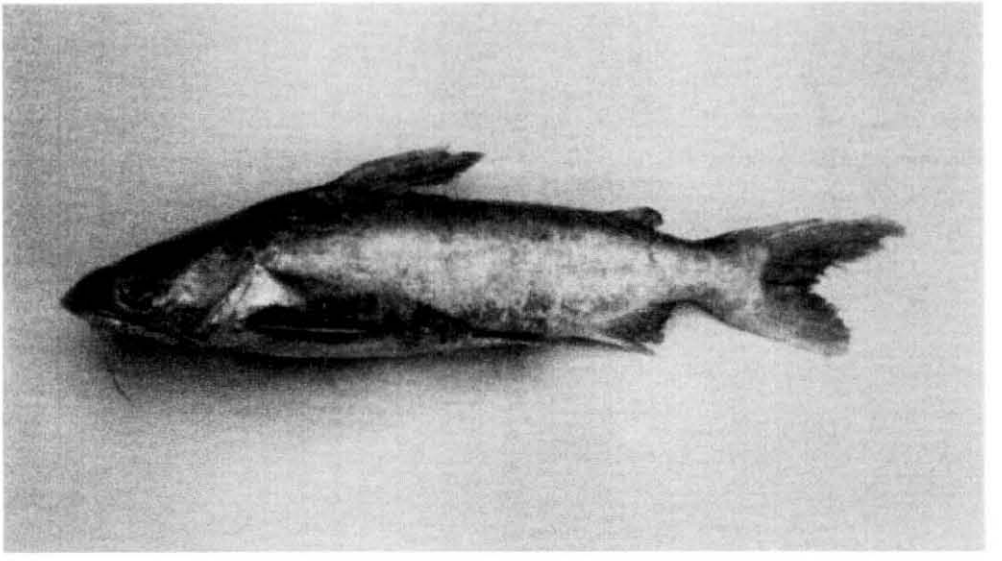
The thesis is arranged into 6 chapters in addition to this general introduction to the subject under Chapter 1. Chapter 2 deals with the biology of the study organisms with reference to their Condition Factor and food and feeding habits. Aspects of the toxicity of the mucus are dealt under Chapter 3. Results of the various experiments to look into the chemistry of the mucus toxins are dealt under Chapter 4, while Chapter 5 deals with the wound healing properties of these toxins. A general discussion forms Chapter 6 wherein

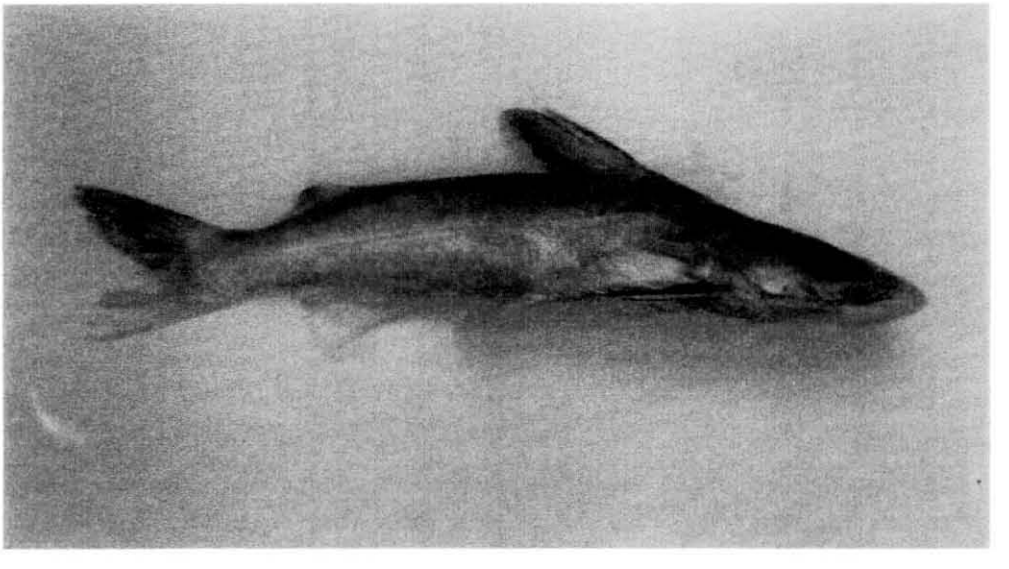
inferences drawn on the basis of results in all the above Chapters are discussed in correlation with one another and in the light of published information. The Chapter also outlines the scope for future work. Chapter 7 gives a list of References cited in this thesis.

Plate 1

a. *Arius dussumieri* (Valenciennes, 1840)

b. *Osteogeneiosus militaris* (Linneaus, 1758)





CHAPTER 2 : BIOLOGY

2.1: Introduction

Widely distributed in tropical Indo-Pacific region, marine catfishes contribute significantly to the fisheries along the Indian Coast, up to the depth of 20-70 m, with particular dominance in muddy bottom areas, either in small aggregation or in large shoals. The adult populations ascend vertically and horizontally (parallel to the coast) for feeding and breeding (Bensam, 1992). Catfish undertake regular diurnal vertical migration; they are found close to the bottom during daytime and at night ascend vertically in the water column and disperse. During their breeding season, they form schools and are found at surface in daytime (Anon, 1987). Trawls, gill nets, hooks and lines, purse seines, and a wide variety of traditional gears are used for their commercial harvest. They migrate from north to south during southwest monsoon and south to north during the post monsoon and are abundant in Gujarat, Maharashtra, Karnataka, Kerala, and the northeast coast of India (Bensam, 1992).

Although they are favorably comparable to other quality fishes in their nutritive value, marine catfish are not treated as quality fishes since, in general, they lack good flavor (Anon. 1987). Therefore, due to the lack of consumer acceptability, they are sold as low-priced fishes. Hence, marine catfishes are considered to be a good, yet cheap source of fish for the preparation of various fish products, like fish *kheema,* sauces, cutlets, fillets, etc. (Dwivedi, 1977). Ariid catfishes have also been found to yield phosphorous, calcium, sodium, and potassium (Kutty *et al.* 1976). Catfish liver

is a good source of vitamin A (Singh and Rege, 1964) and its airbladder can be used to make isinglass (Anon. 1987). Halstead (1970) grouped catfishes along with the other poisonous animals, due to the toxic nature of their spines.

Various workers have reported various species of catfishes worldwide; Gunther (1964) reported 65 species of the genus *Tachysurus* from various parts of British India as against only 23 species of the same genus from the Indian subcontinent reported by Day (1878). Chandy (1953) reported 22 species from various parts of India. FAO (1984) listed 24 species of Ariid catfishes worldwide out of which only 14 were found in India.

The taxonomy of commercially important marine catfishes is still in a state of uncertainty. The nomenclature has suffered a lot of changes brought about by various taxonomists. While some authors have used the genus name *Tachysurus* and hence the family name Tachysuridae, others preferred the name *Arius* and hence the family name Ariidae. Valenciennes (1840), Bleeker (1847), Gunther (1864), Day (1878), Weber and Beaufort (1913), Herre (1953), Jordan (1963), Fisher and Whithead (1974) and FAO (1984) all have used the name *Arius* Valenciennes 1840, whereas Fowler (1941), Chandy (1953), Munro (1955), Tilak (1965), Jayaram & Dhanze (1978 a, 1978 b) and Menon (1979) replaced the name *Arius* Valenciennes 1840 by *Tachysurus* Lacepede 1803. Though all taxonomists agree with the characters by which the genus is identified, the controversy still remains the same.

As the generic name *Tachysurus* was proposed first by Lacepede in 1803, this name is supposed to have precedence over *Arius* Valenciennes 1840 and so Jayaram and Dhanze (1978 a) regarded *Tachysurus* as a valid generic name. However, later for the preparation of FAO Species Identification Sheets

for Western Indian Ocean (FAO, 1984), Jayaram has changed the generic name over to *Arius*, keeping the possible reason with himself. In the present study the generic name *Arius* is used following FAO (1984).

2.1.1: Length-weight Relationship and Condition Factor

In fishery biology, studies on the growth of fish assume special significance. The weight of a fish is a function of length and therefore, these two measurements are closely related. As growth of fish varies from species to species and environment to environment, knowledge of length-weight relationship deserves greater importance from fisheries point of view for a particular area. The knowledge of length-weight relationship helps in estimating stock, in finding out suitable age or length for harvesting, regulating mesh size of nets, and other policy framing for efficient management of a fishery.

According to Le Cren (1951) a knowledge on the length-weight relationship serves two purposes: first, towards describing mathematical relationship between length and weight, so that one of the variable may be converted into the other. Secondly, to measure the variation from the expected weight for length of individual fish or relevant groups of individual as indication of fatness, general well being, and gonadal development, etc.

According to Lagler *et al.* (1962) the growth of the fish represents an increase in three dimensions, whereas length measurements are taken in one dimension. Further it has been found that a fish passes through several distinct stages (Vasnetsov, 1953) of growth, each of which may have its own length-weight relationship (Tesch, 1968). This relationship also lends itself to

comparison of individuals within and between different populations (Lagler *et al.* 1962). Hence the studies on the length-weight relationship of the fish would give a fair idea of the nature of the fish growth (Martin, 1949; Beverton and Holt, 1957; Ricker, 1958; Tesch, 1968).

One of the uses of length-weight relationship is to find out the condition factor. One major concomitant of somatic growth in fish is change in corpulence during life. The change can be great or small, smoothly progressive, intermittent, or cyclically related to breeding (Weatherly and Rogers, 1978). The approach in fishery biology has been to determine the condition factor (also called Coefficient of Condition, Ponderal Index, etc.) with the objective of expressing condition of fish in numerical terms (degree of well being, relative robustness, plumpness or fatness).

2.1.2: Food and Feeding Habits

One of the main aspects of the study on the biology of a fish is to determine its food and feeding habits. Feeding is one of the important activities of a fish as its survival, growth, development, reproduction, etc. take place at the expense of the energy that enters the organism in the form of food. Lagler *et al.* (1962) classified fishes, based on their feeding habits into different types such as predators, grazers, strainers, suckers and parasites. However, the adaptations to the above method are commensurate with the food they eat and are also subject to variations due to ontogenic changes. Changes in the food composition of the fish also take place during its life cycle and such changes also take place in the structure of the feeding and digestive organs (Nikolsky, 1963). Certain biological parameters *viz.* length-weight relationship

and food and feeding habits have been studied in great detail in many groups of fishes such as carps, salmon, elasmobranchs, sardines, mackerels, Bombay duck, sciaenids, polynemids, tunas, seer fishes, carangids, silver-bellies, ribbon fishes, eels, etc.

But the catfishes are rather poorly studied with reference to their length-weight relationship and food and feeding habits, the studies being restricted to those on 9 species of the genus *Tachysurus.*

In the present study an attempt is made to study some aspects of the biology, condition factor based on length-weight relationship and food and feeding habits, and its correlation with the crinotoxicity of two catfishes, *Arius dussumieri* (Black lip sea catfish) and *Osteogeneiosus militaris* (Soldier catfish), which are abundantly available along the Mumbai coast.

2. 2: Review of Literature

2.2.1: Length-weight Relationship

Pantulu (1963) reported the length - weight relationship of *Osteogeneiosus militaris* from Hooghly estuarine system as Log W = -5.6560 + 3.2369 log l with a coefficient of co-relation of this linear regression 0.9933. He tested the value of exponent 'n' against the value '3' and found to be non-significant at the 5% level. He concluded that there was no significant departure in the length-weight relationship of this fish from the cubic equation.

Mojumder (1971) found no significant difference between the length-weight relationships of males and femalses of *Tachysurus thalassinus* from Visakhapatnam coast. He gave a common equation for both the sexes as $W=0.009361\ L^{2.9889}$.

Menon (1979) studied the length-weight relationship for the yolked larva, immature female, mature female and male *T. thalassinus* and found a significant deviation from the cubical relationship. The length-weight relationship of *T. dussumieri* from Mandapam waters was also found to be deviating from the cubic relationship (Anon. 1987). Singh and Rege (1968) determined the length-weight relationship of *T. sona* from Bombay waters and found that the relationship strictly followed the cube law. The length-weight relationship of *T. tenuispinis* from two zones off Visakhapatnam; and length-weight relationship for different sexes were analyzed separately and no significant difference was found in their regressions either for different zones or for different sexes (Dan and Majumdar, 1978). Studies by Roy (1979) on *T. maculatus* had shown that there existed certain changes in various relationships of length and weight and that there was no significant difference as a whole between males and females.

Length-weight relationship of the flat-mouthed catfish *T. platystomus* was significantly different for males and females (Menon, 1984). Das *et al.* (1997) observed significantly different length-weight relationship between males and females of *A. tenuispinis*. Srikrishna (1981) observed that there was no significant difference in the length-weight relationship between males and females of *Osteogeneiosus militaris* off Bombay waters.

2.2.2: Condition Factor

Among mature males and females of *T. thalassinus*, low values of the Relative condition factor (K_n) were observed during the months of April to August, which had coincided with the breeding season of the species.

Similarly, low values of K_n were noticed at 290 mm, which could be correlated with the size at first maturity; the subsequent falls of K_n at 360-370, 450-460 and 510 mm may be indicative of spawning at the second, third and fourth years, respectively (Menon, 1979).

Singh and Rege (1968) found K_n values of *T. sona* to vary at sizes of 240 mm, 345 mm, 475 mm and 525 mm correspondingly representing the spawnings at ages 2, 3, 4 and 5 years.

Dan (1977) observed an increase in the K_n value of *T. tenuispinis* of Visakhapatnam up to May, followed by a steep fall in the subsequent months, which indicated the spawning. The K_n values in relation to size showed three peaks, at 275mm, 335mm and 400mm.

Among the mature fishes of *T. platystomus* a lowering of K_n value was observed in the months of December, January and February, the breeding time of the species. The fall of K_n value at sizes 290 mm, 350 mm, and 380 mm represented the first, second and third spawnings at age 2, 3 and 4 respectively (Menon, 1984).

2.2.3: Food and Feeding Habits

Chaco (1949) had examined the stomach contents of *T. thalassinus* from the Gulf of Mannar and reported that the species was omnivorous. Devanesan and Chidambram (1953) working on the same species from the West coast found sea-cucumber, cuttle fish, small crustaceans, amphipods, prawns, crabs, worms and small fishes in the gut content whereas Suseelan and Nair (1969) found crabs, fishes, prawns, stomatopods, polychaetes and salps in the gut content of the same species off Bombay waters and concluded

that this species was a carnivore and a partial scavenger. Mojumder (1969) confirmed the carnivorous nature of this species from Waltair waters with crabs, prawns, *Squilla* sp. and many demersal fishes found in the gut.

Menon (1979) extensively studied the gut content of *T. thalassinus* from Palk Bay and Gulf of Mannar and found echiurids, crabs, prawns, stomatopods, polychaetes and fishes in the specimens from Palk Bay, and *Philine* sp. crabs, alpheids, prawns, ostracods, amphipods and fishes from the specimens from the Gulf of Mannar.

Devanesan and Chidambaram (1953), Venkataraman (1960), Suseelan and Nair (1969) and Menon (1979) studied food and feeding habits of *T. dussumieri* respectively from Calicut, Bombay and Mandapam and confirmed that this fish was a carnivorous bottom feeder. The food comprised of polychaetes, ophiurids, bivalves, crabs, amphipods, brittle stars, teleosts and seaweeds. *T. jella* is an omnivore and observed to feed chiefly on molluscs, *Lucifer*, prawns, crabs, polychaetes, amphipods, stomatopods and *Squilla* sp. (Devanesan and Chidambram, 1953; Rao, 1964; Suseelan and Nair, 1969).

Menon (1984) found crabs, echiurids, prawns, *Squilla* sp. polychaetes and molluscs in the gut content of *T. platystomus* from Mandapam waters, and concluded that this species was a carnivorous demersal feeder.

Mojumder and Das (1979) observed crabs, prawns, *Squilla,* polychaetes, molluscs and echiurids in the gut of *T. tenuispinis.* Roy (1979) studies the food and feeding of *T. maculatus* from Bombay waters and found ostracods, amphipods, salps, crabs, polychaetes, etc. in the gut content. Devanesan and Chidambaram (1953), Venkataraman (1960) and Srikrishna (1981) studied the gut content of *O. militaris* of West Coast, Calicut and

Bombay waters respectively. They concluded that this fish was a bottom feeder and mainly feeds on polychaetes, molluscs, crabs, prawns, brittle stars and whitebaits.

2.3: Material and Methods

2.3.1: Collection of Samples

Samples of catfishes, *Arius dussumieri* and *Osteogeneiosus militaris* (Plate 1) caught by trawl, long line, gillnet, dolnet, etc. were collected from different landing centers (Versova, Ferrywarf, Sassoon dock) on a monthly basis from March, 1997 to March 1998.

2.3.2: Length-Weight Relationship

Total length (TL) of each collected catfish of either sex was recorded to the nearest mm and its corresponding weight to the nearest gram was also recorded. Length weight relationship was established by fitting the equation of the form

$$W = aL^b \quad \text{.....(1)}$$

where W = Weight

L = Length

a = Intercept

b = Slope

Equation (1) was expressed in the linear form by using logarithms as

$$\log W = \log a + b \log L \quad \text{..... (2)}$$

This linear equation was fitted for each species separately and weights were estimated for different lengths using eqation (1).

2.3.3: Determination of Condition Factor

The Condition Factor was computed as the ratio of observed weight to estimated weight for each fish. The equation used was

$$Kn = W/aL^b \quad \text{..... (3)}$$

where W = Weight of the individual

L = Length of the individual

a and b = Constants from the Length-Weight relation.

2.3.4: Food and Feeding Habit

Generally catfishes are strong carnivorous species, the food item taken by them generally being reported as big organisms that remain intact in shape inside the stomach. Therefore, in the present study quantitative analysis on eye estimation by point method as outlined by Hynes (1950) was adopted to calculate the food items in the guts of fishes.

Samples collected from the different landing centers were brought to the laboratory, cleaned, weighed and measured carefully before dissecting. Upon dissection the condition of the stomach *in situ*, mainly the degree of fullness of the stomach, was recorded and accordingly graded as gorged, full, $3/4^{th}$ full, 1/2full, $1/4^{th}$ full, little full and empty and as per gradation a point of 20, 16, 12, 8, 4, 2 or 0 had been allotted to respective grade of stomach.

The food components found in the guts were grouped into 6 main groups *viz.*, Crustaceans, fishes, molluscs, polychaetes, detritus and miscellaneous. The total points gained by each group of food were scaled down to percentage.

2.4: Results

2.4.1: Length – weight Relationship and Condition Factor

The length-weight rlationship computed for each month, and the condition factor computed therefrom for each month, are presented in Table 1 (*A. dussumieri*) and Table 2 (*O. militaris*). Condition factor in both the cases showed a definitive trend, increasing towards the breeding season and falling sharply immediately thereafter. Observed values ranged between 0.75 (November) and 1.05 (March) in case of *A. dussumieri* and between 0.72 (September) and 1.45 (March) in case of *O. militaris*.

2.4.2: Food and Feeding

The following organisms were found in the gut, which were further grouped under broad heads as:

A. Crustaceans : Prawns, Crabs, Prawn larvae, *Squilla* sp. Alima larvae.

B. Fishes : Teleosts, Fish scales, Fish eggs.

C. Molluscs : Shell pieces, *Phola* sp. *Volida sp.*

D. Polychaetes : *Glycera* sp. *Lumbriconereis* sp. Other polychaetes.

E. Detritus : Unseparable blackish-greenish mass, Stone, Sand and mud particles, other animal source residues.

F. Miscellaneous : Brittle star (*Ophiothrix* sp.), Ascidians, Ostracods, Amphipods, Unidentified matter.

The percentage composition of the various food items as listed above, as also details of the fullness of the stomach, are presented in Tables 3 and 4, and Figs. 1 and 2 respectively for *A. dussumieri* and *O. militaris*.

2.5: Discussion

Relative Condition Factor showed variations on a monthly basis, and appear to be influenced by feeding and breeding activities as had been reported to be the case in many fishes including catfishes (Anon. 1987). Similar results have been reported in all the catfishes studied, *viz. T. thalassinus*, (Menon, 1979), *T. sona* (Singh and Rege, 1968), *T. tenuispinis* (Dan, 1977), and *T. platystomus* (Menon, 1984).

Both the species studied appear to be carnivorous bottom feeders, crustaceans followed by other smaller fishes froming the major food item in the gut contents. Benthic invertebrates such as molluscs and polychaetes followed in importance while sea stars, brittle stars, amphipods, ostracods, etc. (grouped under miscellaneous) formed a minor component. Earlier studies on *Arius dussumieri* (Devanesan and Chidambaram, 1953; Suseelan and Nair, 1969) and Menon (1979) and on *O. militaris* (Devanesan and Chidammbaram, 1753; Venkataraman, 1960 and Srikrishna, 1981) also reveal the two species to be carnivorous benthic feeders, feeding mainly on crustaceans such as crabs and prawns. Relative percentage of the various food items in any given month showed slight fluctuations but there was no major deviation in the overall pattern of composition.

Feeding intensity in each species showed a fluctuating trend monthwise and the maximum feding intensity was during post-breeding in both the cases of *A. dussumieri* and *O. militaris*. In general decreased feeding intensity had been associated with breeding activity in all the catfishes (Anon. 1987). This pattern was observed in *O. militaris* by Srikrishna (1981) wherein during the active period of breeding (March to May) feeding intensity was nil or low and

during the subsequent period was high. The present study also revealed a similar trend in both the species studied; although breeding biology was not addressed to in the present study, the monthwise variations could be compared to the reported breeding seasons of these species.

Both *A. dussumieri* and *O. militaris* are reported to have a prolonged breeding season lasting from May to September (Menon and Muthiah, 1987). The observed monthly variations in the feeding intensity of these two species coincide with such a breeding activity.

Table 1

Length-Weight relationship and Condition Factor of *A. dussumieri*

Month	Length-Weight relationship	Condition Factor
March,97	$- 3.311 L^{2.2877}$	1.05
April	$- 4.06 L^{2.842}$	1.00
May	$- 4.240 L^{2.705}$	0.98
June	$- 2.632 L^{2.042}$	0.95
July	$- 3.529 L^{2.416}$	0.96
August	$- 0.9852 L^{1.3925}$	0.90
September	$- 1.212 L^{1.494}$	0.90
October	$- 3.446 L^{2.378}$	0.85
November	$- 2.574 L^{2.035}$	0.75
December	$- 2.194 L^{1.846}$	0.96
January, 98	$- 2.815 L^{2.086}$	1.05
February	$- 3.870 L^{2.524}$	1.10
March	$- 4.202 L^{2.693}$	1.00

Table 2

Length-Weight relationship and Condition Factor of *O. militaris*

Month	Length-Weight relationship	Condition Factor
March-97	$-4.786L^{2.888}$	1.45
April	$-2.254L^{1.848}$	1.30
May	$-4.529L^{2.788}$	1.10
June	$-5.1988L^{3.061}$	1.00
July	$-5.309L^{3.102}$	0.85
August	$-5.717L^{3.287}$	0.80
September	$-5.393L^{3.142}$	0.72
October	$-5.231L^{3.072}$	0.90
November	$-4.758L^{2.876}$	0.90
December	$-5.010L^{2.989}$	0.95
January-98	$-4.882L^{2.930}$	1.08
February	$-5.429L^{3.158}$	1.17
March	$-5.333L^{3.118}$	1.30

Table 3. Showing percentage composition of food items in the gut content and fullness of stomach of *A. dussumieri*

Food items	Mar,97	Apr.	May.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan, 98	Feb.	Mar.98
Crustaceans	28.22	22.80	29.19	31.29	36.27	40.29	43.29	41.02	30.89	33.26	35.76	37.23	30.71
Molluscs	13.56	18.29	13.53	16.57	22.63	19.47	17.54	14.28	21.50	16.27	14.84	16.88	16.20
Fishes	39.40	42.58	41.82	37.33	13.40	17.21	21.39	27.58	20.49	30.43	27.67	28.93	35.24
Polycheates	12.40	9.14	8.75	4.27	14.28	16.27	11.27	7.89	8.57	3.59	12.22	7.12	17.52
Miscellaneous	5.29	3.72	2.28	5.28	13.07	4.87	3.78	3.56	11.88	5.40	7.23	4.37	4.20
Detritus	1.20	3.51	4.50	5.30	1.24	2.01	2.88	5.63	6.70	11.23	2.32	5.52	6.39
Fullness of stomach	Full	3/4	1/4	Trace	Empty	3/4	Full	1/2	1/4	3/4	Full	1/2	Full

Table 4. Showing percentage composition of food items in the gut content and fullness of stomach of *O. militaris*

Food items	Mar,97	Apr.	May.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan, 98	Feb.	Mar.98
Crustaceans	42.33	30.23	36.23	34.20	32.10	32.40	24.12	30.40	52.52	43.70	38.30	43.58	36.80
Molluscs	15.20	16.26	21.80	20.50	13.63	30.56	37.38	17.86	13.77	15.20	16.32	20.50	18.60
Fishes	25.09	24.07	18.00	20.18	26.78	20.10	26.10	28.40	16.90	20.23	22.18	12.76	19.24
Polycheates	10.47	21.28	11.80	9.25	4.90	9.25	2.80	12.80	13.30	16.08	19.10	16.23	12.43
Miscellaneous	4.3	4.7	10.5	12.23	20.11	3.52	4.20	7.67	2.21	1.20	2.72	4.40	9.23
Detritus	3.00	3.50	2.80	3.70	2.60	4.20	6.30	3.27	1.30	3.87	1.40	2.80	4.20
Fullness of stomach	3/4	Full	1/2	1/4	1/2	Full	1/2	1/2	3/4	Full	Gorged	1/2	Full

Fig. 1: Percentage composition of food items in the gut content of *A. dussumieri*

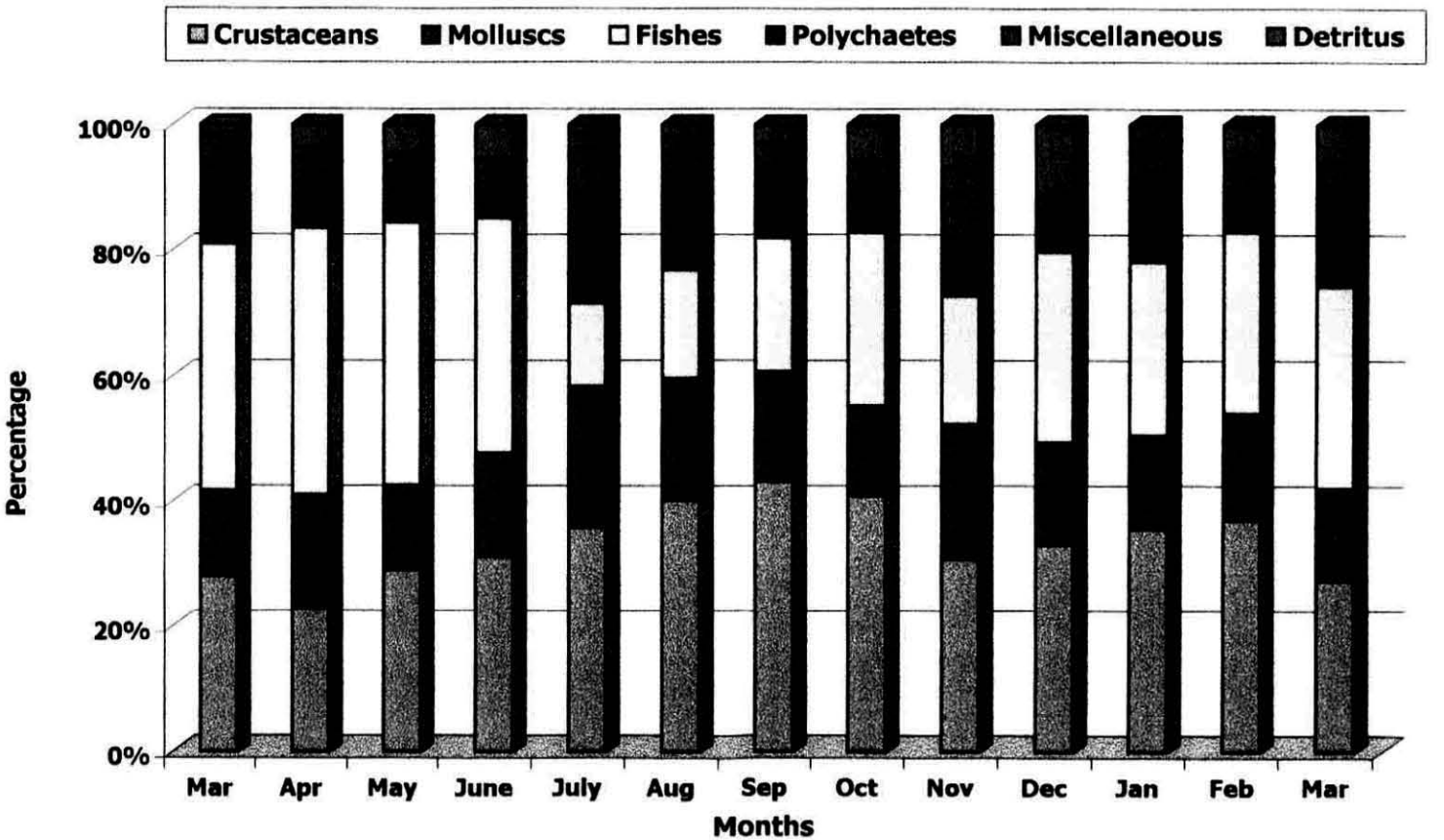
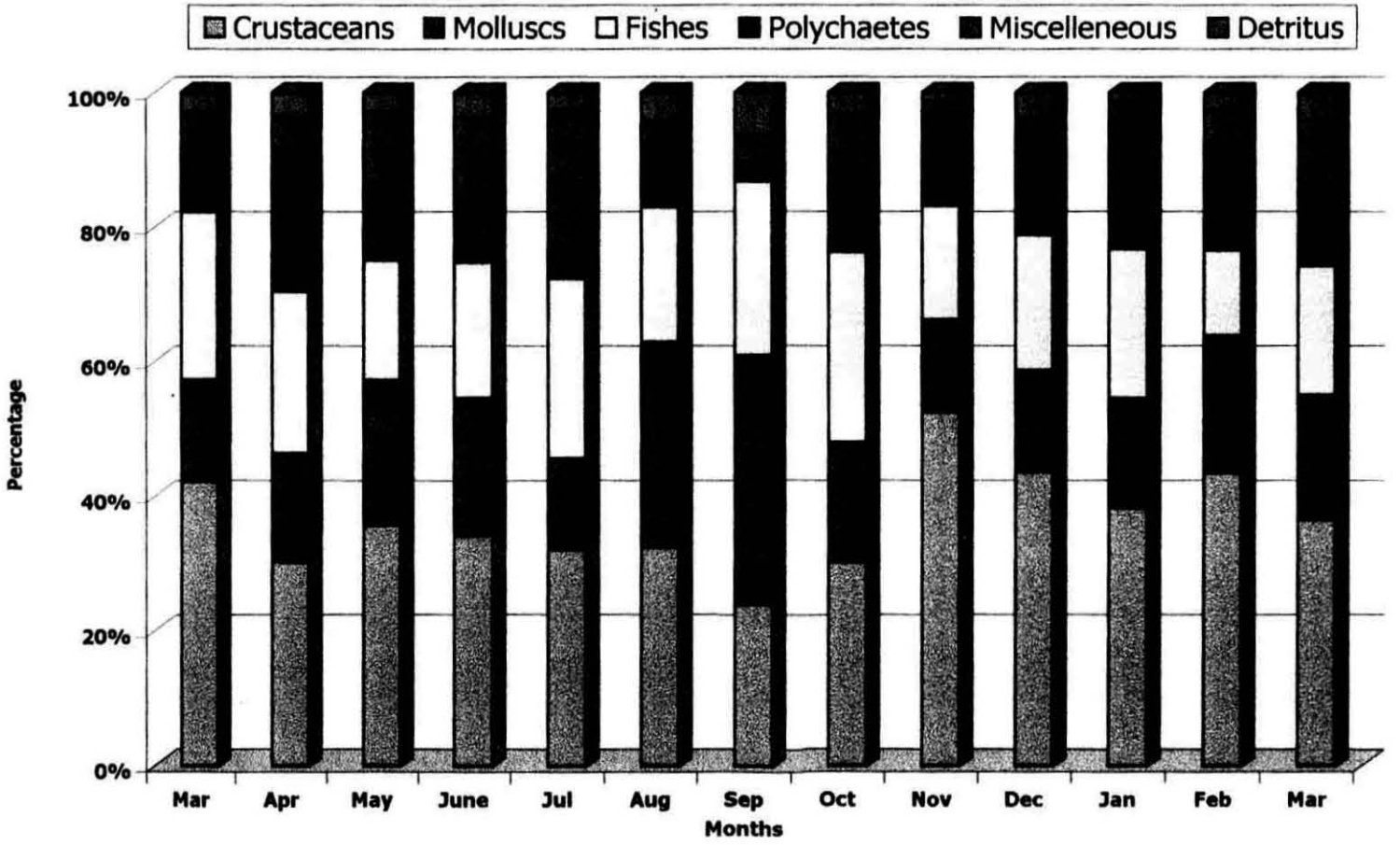


Fig. 2: Percentage composition of food items in the gut content of *O. militaris*



CHAPTER 3. TOXICITY

3.1: Introduction

Man has always been vulnerable to accidental food poisoning because of the presence of many biological toxins in the food consumed. The situation becomes more complicated in the case of seafood, which is considered to be a cheapest source of protein. Sea food poisoning is universal, but is more prevalent in tropical waters, thus affecting most of the developing countries.

A biotoxin from marine organisms usually comes to the attention of biologists because it exerts a striking effect on other organism in the marine community. Many sea creatures can directly injure, envenom and poison their victims and biotoxins are found throughout the entire phylogenetic series of marine fauna. Of all these biotoxins, perhaps less than 1% has been examined for their biological activity (Halstead, 1965).

Most of the marine biotoxins are complex mixture of substances that vary considerably in their bioactivity (Russell and Brodie, 1974). Very little is known of the biological significance of marine biotoxins. However, they appear to serve as defensive or offensive mechanisms in food procurement, or they may be accidentally contracted in the food web of the organism.

3.1.1: Toxigenesis

Toxicity in the marine organism is the result of a progression of biochemical events taking place in the body of target organism. These toxic organisms synthesize various combinations of the atoms of carbon, hydrogen, oxygen, nitrogen, chlorine, sulfur or phosphorous into a biotoxin molecule that

may have extreme complexity and toxicity. The process by which this is accomplished variously is reffered to as "biogenesis", "biosynthesis" or more specifically as "toxigenesis" (Halstead, 1994).

In-depth studies and generation of chemical data suggest that in some cases marine bacteria play a role in toxin biosynthesis. However, synthesis of biotoxins as a result of naturally occuring precursor chemical agents or as the result of human - induced chemical pollutants can also be a possible reason. In all cases, the resulting biotoxins are capable of producing serious public health hazards (Okada and Niwa, 1998).

Each year, an estimated 40,000 to 50,000 people throughout the world die as a result of accidental contact with venomous animals. Venomous animals produce zootoxins in specialized glands that are often associated with a sharp delivery mechanism, such as spines, stings, teeth or other piercing devices. eg. sharks, rays, scorpionfish, weeverfish, stonefish, etc. The venom apparatus may be primarily for killing or paralyzing the prey or may be a purely defensive adaptation. Some venoms also function as digestive fluids (de Wind, 1996).

Halstead (1970) coined the term 'ichthyocrinotoxins' for epidermal secretions of fishes which are toxic but which are not associated with any venom delivery apparatus. They bear glandular elements in the skin that produce toxic secretions that are released in the water, as a defensive weapon. Tachibana (1988) suggested that the crinotoxicity is a type of chemical defense in fishes to evade predation or infection.

The toxic behaviour of ichthyocrinotoxic fishes are comparable to that of amphibians, such as toads and frogs that bear venomous glands in their skin

but lack inoculatory apparatus; the venom serves them for defense purposes, intoxicating predators by contact. Besides serving as a repellent to enemies, epidermal secretions exert, with their toxic metabolites, a most profound effect upon the sea environment, affecting the number and distribution of associated plants and animals, influencing and determining in such a way the character of the biotic community (Maretic, 1988). Ichthyocrinotoxic fishes are generally bottom dwellers and sluggish, and most have habits of 'station-keeping' (Cameron and Endean, 1973).

Ichthyocrinotoxic group of fishes is intermediate between venomous and poisonous fishes (Halstead, 1970). About 50 species of fishes belonging to 13 families were reported as being ichthyocrinotoxic (Cameron and Endean, 1973).

Catfishes are known to contain venom and toxin that can be found either in the venom glands in the dorsal and pectoral spines or in the skin seccretions of the body (Othman *et al.* 1993). Catfish skin toxin and the venom from their dorsal and pectoral spines may cause a menacing sting and although these stings are often innocuous, severe tissue necrosis may occur. Symptoms are caused by hemolytic, dermonecrotic, edematic, vasospastic and lethal components of the venom and skin toxins (Mann and Werntz, 1991). Shiomi *et al.* (1987) suspected symptoms induced by catfish toxin was due to the combined effect of both spine and skin secretion.

Considerable work has been done worldwide on the spine toxin of stonefish, scorpion fish, weeverfish, lionfish, soldierfish (Auerbach, 1988) whereas work on catfish spine and skin toxin is meager and include those on freshwater fishes, viz. *Heteropneustes fossils* (Bhimachar, 1944; Dutta *et al.*

1982, Lahiri *et al.* 1983, and Deo and Venkateshvaran, 1999), ictalurid catfishes (Birkhead 1967, 1972), the estuarine catfish, *Plotosus linetus* (Shiomi *et al.* 1986,1987,1988), *Plotosus canius* (Auddy *et al.* 1994), and marine catfish *Arius thalassinus* and *A. bilineatus* (Al-Hassan *et al.* 1982, 1983, 1985, 1986, 1987; Alnaqeeb *et al.* 1987).

However, information on toxicity of Indian mairne catfishes is scanty, and it is in this backdrop that the present work was taken up to establish the crinotoxic nature of the two Indian marine catfishes *A. dussumieri* and *O. militaris* commonly available along the north west coast.

3.2: Review of Literature

Fish have a number of accessary structures interspersed among their skin connective tissues that greatly extend the apparent simple function of the body covering (Junqueira *et al.* 1970). Among the most evident of these structures are the mucus cells that produce the slimy, lubricating secretion covering the external surface of the skin, so evident when holding the fish.

3.2.1: Role of Fish Mucus

The range of roles which fish mucus is known to play is very large (Ellis, 1981). Several important biological functions have been ascribed to the mucus layer of fish. It has been implicated as a lubricant, involved in osmoregulation and locomotion, (Negus, 1963; Rosen and Cornford, 1971; Bernadsky *et al.* 1993) and has a mechanical protective function (Cameron and Endean, 1973). Mucus may play a role in the prevention of colonisation by parasites, bacteria, and fungi and thus act as a chemical defence barrier (Jakowska, 1963).

Furthermore, specific immunoglobulins (IgM) have also been reported in the mucus layer of some fish species (Fletcher and Grant, 1969; Bradshaw *et al.* 1971). The presence of antibodies and specific anti-pathogenic factors in mucus would be beneficial to the host (Ingram, 1980). Mucus is also a source of natural agglutinins and lysins (Spitzer *et al.* 1976; Di Conza and Halliday, 1971). Fletcher and Grant (1969) detected complement and lysozyme in fish skin mucus. Ramos and Smith (1978) observed CRP-like substances in epidermal mucus of fish. Functions of fish mucus in ion regulation (Shephard, 1982) ammonia excretion (Wright *et al.* 1989), and filter feeding (Sanderson *et al.* 1991) are well established. Fish are able to alter the amount of mucus secreted by the epidermal cells (Jakowska, 1963). Stress, as a result of infection or handling, is known to cause an increased mucus production in fish (Pickring, 1976). It is most likely that the level of mucus secretion reflects the degree of initial resistance to invasion by variety of potential infective agents. Epithelial mucus contains both protein and carbohydrate moieties and is similar in structure of the mucins found in other animals (Ingram, 1980). The chemical composition of fish mucins, especially of the glycoproteins, has been investigated in several species (Fletcher and Grant, 1969; Pickering 1976; Wold and Selset, 1977). Shephard (1994) reviewed the functions of fish mucus, which is represented diagramatically in Fig. 3.

3.2.2: Histology of Fish Skin

Skin has traditionally been regarded as a passive rind that holds the animal together. The skin surface of teleost fish has on it a varying population of living organisms that comprise the biota of its ecosystem but in contrast to

the higher vertebrates the skin surface of teleosts is itself a living tissue surface. The teleost skin develops cyclical and specific changes in its structure and secretions during growth and maturity that are dependent on both internal and external stimuli and are the major factors for change in the total system (Roberts and Bullock, 1980).

Fish skin, like that of other vertebrates, is a dynamic, versatile organ and consists of a hypodermis, dermis and epidermis (Roberts and Bullock, 1980). An additional component is the cuticle, a layer of mucoid and formed squamous elements which invests the surface but varies considerably in thickness depending on its location of the body surface and the state of maturity and health of the fish (Whitear, 1970). The cuticle is in fact an ill-defined amorphous layer of varying thickness derived from the epidermis. It contains some formed elements such as fibres and organelles from desquamated epithelical cells, in a substrate comprised in part of mucus from the mucus cell component of the epidermis and in part from malpighian cell secretions and cell fluids of sloughed cells. In some cases, the cuticle may contain some other formed elements such as melanin granules and leucocytes. The cuticle has a variety of physiological functions. It has been shown to reduce the drag of an animal swimming through the water (Rosen and Cornford, 1971). It provides a slippery surface to facilitate escape from the grasp of predators and it provides an inhospitable surface in the healthy animal for colonisation by opportunistic saprophytes or pathogens.

The epidermis in fish is a stratified epithelium and also varies in thickness between species (Bullock and Roberts, 1975). The epidermis has two developmental potentials in fish: mucogenesis and keratinization. In most of

the fishes, the epidermis is mucogenic, while in some fishes, epidermis on the general body surface and at specialized and localised structures *viz.* breeding tubercles, epithelium of lips and associated structures, adhesive apparatus, etc. is found to be keratinzed (Mittal and Banerjee, 1974, Mittal and Whitear, 1979), which is considered as a characteristic feature of the epidermis of land vertebrates.

The basic structural unit of fish epidermis is epithelial cells or malpighian cells or polygonal cells, which is rich in cytoplasmic fibres and undergoes a degree of degeneration as it matures. The epidermis is capable of mitosis right on its outermost layer (Bullock *et al.* 1978). It always contains apocrine mucous cells in varying numbers, which are unicellular glands, and secrete different mucoid types but in addition, in various species there may be other types of gland cells such as club cells and the sacciform cells. Many of the club cells secrete a fright stimulant (Von Frisch 1941) or, in the case of Ostraciidae, a potent neurotoxin (Thomson, 1969). Whitear and Mittal (1983) suggested that the primary function of the club cell is protecive and the release of specific fright stimulant of Ostraciidae is a secondary phenomenon.

Involvement of club cells in the secretion of active hemolysin and hemagglutinin in the eel skin was well demonstrated by Suzuki and Kaneko (1986). Various leucocytes are present in lower epidermis; besides this, eosinophilic granular cells of unknown funtion are also found in the epidermis of many species. There are various types of nerve endings within the epidermis and these vary in complexity from highly specialised mechanoreceptors, the neuromasts, to relatively simple chemoreceptors (Whitear, 1971). The surface cells of the epidermis are generally flattened and with prominent perinuclear

organelles. They are connected by desmosomes with a junctional complex between each cell. The most important feature of fish epidermis is the series of raised whorls or ridges, into which the outer surface cell membranes of the eipthelial cells are arranged, which is probably an artefactual representation of cuticular material which has remained firmly attached to the ridges during the process of fixation, studied extensively by electron microscopy, and reffered as 'fine fibrillar fuzz' (Henrick and Matoltsy, 1968, Schliwa, 1975) or glycocalyx (Schwerdtfeger and Bereiter-Hahn, 1977).

The dermis is made up of two layers, stratum spongiosum and stratum compactum. The upper layer of dermis is stratum spongiosum, which is a complex layer comprising the scales, pigment cells, mast cells and the fine argyrophilic fibres binding the epidermis. The stratum compactum is a dense layer composed of collagen and penetrated by vessels and nerves serving the spongiosum. The hypodermis is richly endowed with vessels and hence, like the stratum spongiosum, it is a relatively loose tissue (Roberts and Bullock, 1976).

3.2.3: Origin of Crinotoxin

Evolutionary lines of secretory epidermal cells divide into two lines of cells, mucus cell stem lines and proteinaceous cell stem lines (Quay, 1972). In fishes, mucus cells are found in the form of goblet cells, within which the secretory product is stored in the form of globules. These goblet cells are widespread in occurrence and are usually the dominant secretory elements in the epidermis (Lagler *et al.* 1962). Mucus cells produce slime that helps in reducing turbulence during swimming (Rosen and Cornford, 1971).

Reed (1924) and Birkhead (1967) observed proteinaceous (clavate) cells as the source of origin of crinotoxin in ictalurid catfishes. Reed (1924) further suggested that the proteinaceous secretion of clavate cells of the epidermis of catfishes had a deleterious effect on the fungus *Saprolegnia.* Cameron and Endean (1972) postulated that the large clavate proteinaceous cells of epidermis are the source of crinotoxins in Australian batrachoidids. Cameron and Endean (1973) found a positive relationship between reduction of squammation and production of proteinaceous crinotoxin and suggested that the ichthyocrinotoxic fish was an evolutionary stage of venomous acanthotoxic fish.

3.2.4: Toxicity

A large number of species of fish have been reported to be ichthyocrinotoxic (Halstead, 1970). Prokhoroff (1884) was the first to observe crinotoxic nature of certain fishes, which, when kept together with other noncompatible species of fishes, caused the latter's death.

Skin toxins (crinotoxin) have been demonstrated for at least some species of the following families of tropical marine fishes: trunkfishes (Ostraciidae), soapfishes (Grammistidae), gobies (Gobiidae), soles (Solidae), toadfishes (Batrachoididae) and clingfishes (Gobiessocidae). Possible toxic and repelling structures have also been reported from skin of puffers (Tetrodontidae), marine catfishes (Plotosidae and Ariidae), filefishes (Monocanthidae), scorpionfishes (Scorpaenidae) and moray eel (Muraenidae), (Halstead, 1970); Hashimoto, 1979; Randall *et al.* 1981; Shiomi *et al.* 1986; Al-Hassan *et al.* 1985).

Clarke (1918) was the first to report that the gelatinous material of the labial glands and buccal cavity of the trunkfish, *Lactophrys bicaudilis* was highly poisonous. Thomson (1964) isolated a crude toxin from the boxfish *Ostracion lentiginosus* which, when injected i. p. into albino mice @ 0.2 mg/g of body weight, caused ataxia, labored breathing, coma and death quickly. He also found this crude toxin to be hemolytic, and heat stable. Boylan and Scheuer (1967) isolated the pure toxin of these boxfish in crystalline from and named it 'Pahutoxin', after the Hawaiian name of the fish, Pahu. They obtained the toxin by putting the fish in containers of distilled water where they released copious amount of toxic secretion.

Toxic stress secretions of the boxfish *Ostracion meleagris* were highly toxic to marine fishes and also caused hemolysis and hemagglutination on fish erythrocytes (Thomson, 1969). Goldberg *et al.* (1982) isolated a toxin from the mucus secretion of smooth trunkfish, *Lactophrys triqueter* and calculated LC_{50} of 3.5 ppm using mosquitofish as the test organism. They found the minimum lethal concentration to be 2 ppm for mosquitofish. Fusetani and Hashimoto (1987) studied hemolytic nature of the two fractions of mucus secretion of the Japanese boxfish, *Ostracion immaculatus*; the first, major hemolysin showed a hemolytic activity of 2.07 Saponin Units (SU)/ mg and the minor hemolysin exhibited a hemolytic activity of 1.87 SU/mg towards rabbit erythrocytes. Lyophilized samples of the aqueous mucus extracts from five species of trunkfish, *Aracana aurita, A. ornata, Ostracian cubicus, Rhinesomas reipublicae* and *Strophiurichthys robustus* were tested for ichthyotoxicity using mosquitofish as the test organism. *A. aurita* mucus tested at a concentration of 100 ppm resulted in no death of the test fish after 8 hours. *A. ornata* mucus

tested at 15 ppm resulted in three deaths out of six fish after 2 hours of exposure and all six fish died after 4 hours. Mucus of *Rhinesomus reipublicae* tested at 12 ppm resulted in the death of four out of six fish after 2 hours and after 4 hours all the six test fish died. In all cases, a control group of six fish, tested under identical conditions, survived after 8 hours (Goldberg *et al.* 1988).

Liguori *et al.* (1963) studied the skin secretion of the soapfish *Grammistes sexlineatus* and found it toxic to top minnows. Maseretski and Castillo (1967) tested a crude solution of soapfish, *Rypticus saponaceus* by injecting it i.p. into mice and found it to be toxic. They further assayed the toxicity using freshwater guppies as test organisms. They observed that immediately after addition of the skin extract into the container, the guppies underwent a period of motor excitation or hyperactivity, gradually become quieter, and died after 20 to 30 minutes.

Hashimoto and Oshima (1972) separated three components of grammistins, A, B & C from the skin secretion of soapfish *Pogonoperca punctata* by chromatography and tested for hemolytic activity towards 2% rabbit blood cell suspension. They observed that Grammistin A and C had a comparable activity of about 1.7 SU/mg, whereas grammistin B was more than twice as active. Oshima *et al.* (1974) seperated grammistins from four species of soapfishes *P. punctata, G. sexlineatus, Diploprion bifasciatum,* and *Aulacocephalus temmincki* and compared them for hemolytic activity. They observed that the hemolysin of *D. bifasciatum* was different from others with 0.21 SU/mg.

Hori *et al.* (1979) observed ichthyotoxic and hemolytic activity in mucus extract of clingfish *Diademichthys lineatus*. They also demonstrated a special

type of secretory cells in the epidermis of the fish. Shiomi *et al.* (2000) isolated skin toxin of soap fish *G. sexlineatus* in aqueous acetic acid and butanol and assayed for LC_{50} against killifish. The LC_{50} value of GS1 and GS2 fractions were 1.91 µg/ml and 0.19 µg/ml respectively. When assayed for hemolysis, the GS2 fraction showed a higher activity of 1,470 HU/mg towards horse blood, followed by rabbit blood at 780 HU/mg.

Working with butanolic skin extracts of toadfish *Opsanus tau,* Nair *et al.* (1982) observed death in silversides and killifish. The Red Sea flatfish (Moses sole) *Pardachirus marmoratus*, secretes a skin toxin that functions as a defense against shark predation (Clark, 1974; Clark and Chao, 1973). Primor and Zoltkin (1975) found the skin secretion of the flatfish *Pardachirus* to possess ichthyotoxic and direct hemolytic actions; the ichthyotoxic activity was found to be 32 and 210 µg per LD_{50} units for the fresh and lyophilized secretion, whereas hemolytic activity was 9.0 and 66.0 g (dry weight)/HU for the fresh and lyophilized secretion. They concluded that the gill membrane was the primary target of the toxic factors. Primor *et al.* (1978) isolated a peptidic shark repellent, pardaxin from *P. marmoratus*. Studies on the genus *Paradachirus* have shown a diversity of various types produced by toxic secretion or by its isolated toxin (pardaxin) in biological membranes from various origins, red blood cells (Primor and Lazarovici, 1981), nervous tissue (Primor, 1984), and iontransporting epithelia (Primor, 1983). Moran *et al.* (1984) calculated LD_{50} of skin secretion from *P. marmoratus* against killfish to be 2.8 mg. Lazarovici *et al.* (1986) purified two types of pardaxins, PX-I and PX-II from the secretions of the Red Sea Moses sole *P. marmoratus*, which had

toxic, cytolytic, and pore forming factors. They found that PX-I was more toxic, cytolytic and active in membrane pore formation than PX- II.

The lyophilised powder of toxic secretion of *P. pavonius* was suspended in aqueous ammonia and was precipitated by adding acetone. Both the precipitate and the supernatant liquid were active in ichthyotoxicity assays and were hemolytic to erythrocytes (Tachibana *et al.* 1984).

Tachibana *et al.* (1985) seperated six steroids, pavonins 1-6, from the defense secretion of the sole *P. pavoninus* and found all of them had strong ichthyotoxic and hemolytic activity. The LD_{50} of 8.5 µg /ml was recorded by them against killifish.

Ligouri *et al.* (1983) found the extracts of the mucus secretion of the soapfish *Grammistes sexlineatus* to be antibiotic against *Escherichia coli* and *Candida albicans.* Takahashi *et al.* (1887) reported bacteriolytic substances in the skin mucus of yellow tail, *Seriola quinqueradiata* and suspected that this activity was due to the presence of various enzymes. The purified fractions of skin mucus of Ayu had shown bacteriolytic activity against *Micrococcus lysodeikticus* and *Vibrio anguillarum* (Itami *et al.* 1987). Skin secretions from *Cyclichthys spinosus, Sphaeroides spengleri* and *Diodon hystrix* were tested on crustacean nerves, sea urchin eggs and mouse eruthrocyte suspension to verify neurotoxic and cytolytic activites. *C. spinosus* and *D. hystrix* secretions induced transient depolarizations with 0.16 mg and blocked crustacean nerve conduction after prolonged exposure. Both secretions had cytolytic effects and when applied to sea urchin eggs they caused cleavage inhibition and anomalies in a dose-dependent manner (Malpezzi *et al.* 1997).

Noguchi *et al.* (1971) reported the toxic nature of skin of goby, *Gobius criniger* by conducting mouse bioassay. The skin toxin was extracted with hot methanol and slightly acidulated with acetic acid.

The mucus secretion of moray eel *Lycodontis nudivomer* was collected with gauze in 0.02M phosphate buffer solution (pH7.5) and purified into active fractions by chromatographic techniques and finally tested for lethal hemolytic and hemagglutinating activity. The hemolytic activity was as high as 150 SU/g of mucus. The hemolytic and toxic activities were observed in the same fractions. Hemagglutinating activity was also observed when tested towards RBC of human and rabbit (Randall *et al.* 1981). Upon histochemical examination they suspected that the clubbed cells of epidermis were responsible for the hemolytic - toxic components.

Suzuki (1985) extracted skin secretion of Japanese eel *Anguilla japonica* in 0.0099M Vernol buffer (pH7.4) and assayed it for hemolysis and hemagglutination against erythrocytes of human, sheep, rabbit, chicken, carp and eel. He observed that the mucus extract had very potent activity against rabbit erythrocytes with titers of 256-1,024 but not in the case of chicken, carp and eel erythrocytes. Agglutination with the mucus extract was very active against rabbit and human B type erythrocytes, and also against erythrocytes of sheep, chicken, carp and eel.

Shiomi *et al.* (1990) intravenously injected crude extract of the skin secretion of *Anguilla japonica* into mice and found the LD_{50} to range from 6.2 to 22 mg mucus/kg. The symptoms recorded were convulsion and jumping, and the time taken for death was 20 minutes in mice. They also tested this mucus extract for hemolysis and hemagglutination activity and observed that

the toxin was not hemolytic against rabbit erythrocytes, but the hemagglutinating activity was well pronounced.

A souluble skin secretion extract from *Plotosus canius* was found to be toxic to mice with an LD_{50} of 7.0 µg/g body weight (Othman *et al.* 1993). It also exhibited hemolytic activity of 71 HU/mg protein. Upon chromatography, the components were found to have hemolytic activity and a toxicity of 0.02 µg/g body weight. Studies on neuromuscular transmission and myotoxicity on rat phrenic hemidiaphragm and chick biventer cervicis revealed that the toxic component (plotoxin) had completely blocked the muscle twitches in both the case by indirect stimulation, confirming that plotoxin exerts effects presynaptically without affecting the muscle contracatile apparatus (Othman *et al.* 1993).

Working with the oriental catfish *Plotosus lineatus* Shiomi *et al.* (1986) reported hemolytic, lethal, and edema-forming activities in the skin secretion. Agitated behavior, increased heart rate, jumping and convulsion were observed in mice immediately after injection of lethal doses, and finally mice died of respiratory arrest within 30 minutes to several hours. Further purification of the skin secretion by various chromatographic methods produced one lethal factor that had a LD_{50} of 0.71 mg/kg body weight of mice upon i.v. injection, and a strong edema forming activity; the minimum dose inducing 130% edema ratio was as small as 0.89 µg. (Shiomi *et al.*1987). Antiserum was raised against toxin I, and immunodiffusion tests were conducted; the antiserum formed a precipitin line with toxin I but not with toxin II or hemolysin, suggesting that toxin I was antigenically distinguised from both toxin II and hemolysin. By

immunocytochemical tests, it was also confirmed that the source of toxin I was club cells (Shiomi *et al.* 1988).

Intravenous injections of the soluble skin secretions (toxin) into marginal ear vein of rabbits resulted in acute respiratory distress, convulsive movements and death within seven minutes (Al-Hassan *et al.* 1985); the lethal dose in rabbits (LD_{50}) was 1.5 mg protein/kg body weight and at a dose of 4 mg/kg, the rabbit generally died in less than one minute. They also reported a significant increase in total plasma lactate dehydrogenase and the hepatic and cardiac derived isozymes and in glutamic-oxaloacetic and glutamicpyruvic transaminase activities in animals receiving toxins. Major time-dependent toxic effects were noted on the plasma, heart and liver of test animals (Al-Hassan *et al.* 1985, 1987). The epidermal secretion contains esterase activity (Thomson *et al.* 1984), along with hemolytic, plasma clotting and red blood cell agglutinating activities. Thulesius *et al.* (1983) demonstrated potent smooth muscle contracting activity in both the gland and skin secretions. The constrictor effect of the venom was reversible *in vivo*, while that of the skin secretion was irreversible and resulted in death in rats and guinea pigs.

Extensive hemostasis of blood follows treatment of rabbit with the epidermal extract; animals given a lethal dose of 4 mg/kg had massive clot throughout the lungs, heart and blood vessels at the time of death (Al-Hassan *et al.* 1985). Sublethal dose induced major clotting in the lungs and granulation of the liver. Low doses, less than 0.5 mg/kg, caused no visually apparent symptoms. However, levels between 0.5 and 1.0 mg/kg caused observable trauma and significant levels of clotting in the lungs, but most treated animals quickly recovered (Al-Hassan *et al.* 1985). The lethal effects of the gel

secretion can be effectively blocked by pretreatment of the animals with either indomethacin or hydrocortisone both of which inhibit formation of prostaglandins, confirming that production of prostaglandins has a major role in the toxic responses. Further, elevated levels of 6-Keto-PGF1α and thromboxane B2 were found in the serum of treated rabbits with a sublethal dose of the extract (Al-Hassan *et al.* 1986*a*). A galactose specific lectin isolated from the same fish had a strong agglutinating activity towards various red blood cell types (Al-Hassan *et al.* 1986*b*).

Al-Lahham *et al.* (1987) reported one additional protein and an enzyme, both of which had hemolytic activity against various RBCs of different animals. They also observed that these hemolytic factors were not toxic to fish and did not cause lethality when administered i.v. to rabbits.

Intravenous injection of partially purified clotting factor into rabbits, at levels equal to, or greater than that contained in a lethal dose of soluble gel extract, had no apparent effects on the rabbit (Al-Hassan *et al.* 1986*a*). Ali *et al.* (1989) found that epidermal secretions of *A. bilineatus, A. tenuispinis,* when injected i.v. into rabbit, were equally toxic at a dose of 2 mg/kg, death occurring within seven minutes, but *A. thalassinus* preparation was not lethal at the dose of 2 mg/kg. Secretions from all three catfish induced changes in plasma enzyme levels indicating heart and liver damage, and also induced changes in blood glucose and chloesterol levels. Alnaqeeb *et al.* (1989) studied the toxic effect of skin toxin (dose 2 mg/kg i.v. into rabbit) histopoathologically on lung, liver, heart and kidney and concluded that lung and liver of treated rabbits were adversely affected with tearing and thickening of tissues and blood clots, while heart and kidney tissues appeared unaffected. They also

studied the blocking of lethality by treating rabbits with indomethacin prior to the injection of toxin. Such rabbits pretreated with indomethacin showed significant reduction in the damage of lung and liver observed after injection of skin toxin. Purified toxic factor from *A. bilineatus* epidermal secretions exhibited lethality, when injected into the marginal ear vein of rabbit, with the LD_{50} equal to 0.045 mg/kg. The symptoms of toxicity observed were labored breathing, agitation and convulsions and death occurred within five minutes. Analysis of plasma levels of lactate dehydrogenase, glutamate-oxaloacetate tranasminase and glutamatepyruvate transaminase in injected rabbits indicated that the skin toxin caused cardiac and liver damage to the animals (Thomson *et al.* 1998). Low levels of proteolytic activity were found in the soluble gel protein fraction of *A. thalassinus*. However, fractionation of the protein into 20% to 40% ammonium sulfate precipitate removed inhibitors and enhanced protease activity was noted. Phosphatase activity was also found to be very low but lipase, phospholipase or nuclease activities were absent (Al-Hassan *et al.* 1982, 1985).

Auerbach (1988) described nausea, vomiting, dysentery, diarrohoea, tenesmus, abdominal pain, and weakness as symptoms of ichthyocrinotoxicity in human beings. In some cases contact with certain mucus extracts was reported to cause irritant dermatitis. Therapy for the crinotoxic intoxication is symptomatic and supportive.

3.2.5: Structure of the Spine

Bhimachar (1944), Norman and Greenwood (1975), Lagler and Bardach (1977) and Halstead (1980) described the structure of the catfish spine.

Venomous catfishes bear a single stout spine immediately in front of the soft-rayed portion of the dorsal and pectoral fins, associated with the axillary glands. The spine is enveloped by a thin layer of skin called integumentary sheath, containing glandular cells, which are more concentrated at the anterolateral and posterolateral margins of the sting. The spines also bear a series of recurved teeth, which are capable of producing a severe laceration of victim's flesh thereby facilitating absorption of the venom and subsequent secondary infection including gangrene and tetanus. Transmission of venom is due to the spine action; when the spine is erected, the skin and the poison gland exert pressure and the poison is ejected through a temporary rupture of the skin at the tip of the spine into the wound. The venom, which is a poor antigen, contains vasoconstrictive, dermatonecrotic and other biogenic fractions (Calton and Burnett, 1975) circulate in the body with blood.

3.2.6: Venom Apparatus

Bottard (1889) was one of the first to describe the venom apparatus in catfishes. Reed (1900,1906,1907,1924) and Reed and Loyd (1916) reported the venom organs in freshwater catfish belonging to genera *Schilbodes* and *Noturus*. According to their findings, the venom apparatus of these freshwater catfish consists of dorsal and pectoral stings and axillary glands. The axillary glands are situated in the axilla and open outside by a pore. Pawlosky (1927) described the venom apparatus of the members of the genus *Plotosus*. Citterio 1995) described the microscopic anatomy of the venom apparatus of *Ictalurus catus*; its axillary gland was similar to that of *Schilbodes* and *Oturus,* and was also found to be the main site of toxin production. Venom organs of the Indian

catfish, *Heteropneustes fossilis* and *Plotosus arab* was described by Bhimachar (1944). He claimed that the axillary glands were absent in both the fishes and the poison secretion was from the basal epithelial cells of the epidermis covering the spine. Tange (1955) reported that the dorsal stings of *P. lineatus* consist of a central canal with a proximal and distal opening. The venom glands were found to lie beneath the sheath all along the margin of the spine. He also found that the axillary gland was surrounded with a thick capsule of connective tissues. The pectoral spine is provided with an excretory canal that opens on its dorsal side through the small pore. Al-Hassan *et al.* (1987) reported the presence of a well-defined venom gland and axillary pore associated with tissues at the base of the spine in the Gulf catfish *Arius thalassinus.*

3.2.7: Toxicity of Spine

The painful nature of wounds resulting from catfish spines have been described by many workers, notably Pawlosky (1927), Bhimachar (1944), Halstead (1965), Birkhead (1972), and Al-Hassan *et al.* (1994).

Tyoshima (1918) observed neurotoxic and hemotoxic activities in the spine extract of *P. anguillaris.* Human deaths have resulted from the stings of *P. lineatus* (Pawlosky, 1927). Birkhead (1972) found neurotoxic and hemolytic activity in twelve ictalurid and ariid catfishes. He injected spine extracts from both the genera in Gambusia and symptoms observed included necrosis edema, hemorrhage, chromatophore expansion and mortality. He suggested that the active compounds of the spine extracts were proteinaceous in nature. Bhimachar (1944) reported both neurotoxic and hemolytic properties of the

venom from *H. fossilis*; subcutaneous injections of glycerated spine venom of the fish resulted in hypoactivity, tetanic convulsion and death in frog within 15 to 20 minutes and also hemolysed washed RBCs of horse and cow. Lahiri *et al.* (1983) found six separable protein fractions in crude spine venom, five from skin extracts and four from the serum of *H. fossilis* by electrophoresis.

When the spine venom of *Arius thalassinus* was tested for toxicity in small laboratory animals by subcutaneous or i. p. injection at levels of upto 20 mg protein/kg body weight, only minor irritation was observed whereas i.v. injection of venom into rabbits did not cause lethality at levels upto 10 mg protein /kg body weight but the levels of plasma enzymes were altered (Al-Hassan *et al.* 1986a, 1987). They also found hemolytic, neurotoxic and hydrolytic activities in the spine venom of the same fish. Auddy *et al.* (1994) reported that *P. canius* venom increased the blood pressure in mice. The LD_{50} in mice was found to be 3.9 mg/kg. The venom upto a concentration of $7.5x^{10-3}$ g/ml did not show any hemolysis on erythrocytes. Dutta *et al.* (1982) found hypotensive activity in the crude spine extract of *H. fossilis* whose i.v. LD_{50} in mouse was 5.01 mg/kg; it was nonhemolytic to washed human erythrocytes.

Al-Hassan *et al.* (1987) reported 175 injuries by Gulf catfish *A. thalassinus* in human beings over a six-year period. The observed symptoms were severe instantaneous pain accompanied by accelerated heart rate, local paralysis, elevation of body temperature, nausea and edema while gangrene was also noticed in some cases. Wounds generally healed within a few days, but in certain cases months were required, but deaths were not reported in any case.

Fisher (1978) described the envenomation of catfish sting as painful wound, characterised as stinging, thrombing, or scalding and the pain radiating. A mild envenomation may produce pain for 30-60 minutes. Large tropical catfish may apparently cause pain for upto 48 hours. The soft tissue surrounding the wound becomes ischameic in appearance, with cyanosis, reactive hyperamia and edema. Systemic effects include local muscle spasm sweating, and fasciculations.

Symptoms less frequently observed were peripheral neuropathy, lymphoedema, lymphadenopathy, lymphangitis, weakness, syncope, hypotension, respiratory distress and even death Halstead *et al.* (1953). Auerbach (1988) suggested that the sting of the marine catfish was more severe than that from freshwater catfish.

3.3: Material and Methods

3.3.1: Collection of Fishes

Specimens of catfishes, *Arius dussumieri* and *Osteogeneiosus militaris* caught by trawl, long line, gillnet, etc. were obtained from the local landing centers (Versova, Ferrywharf and Sassoon Dock) on a monthly basis from March 1997 to March 1998, brought to the laboratory in ice, and immediately preserved at -20^0 C.

3.3.2: Extraction of Crude Toxin

Crude toxin was extracted following Al-Hassan *et al.* (1982, 1986). Twelve fishes from each monthly collection were thawed to room temperature whereupon mucus was scrapped with a dull blade in phosphate buffer saline

(PBS) pH 7.5, and was homogenized. The homogenate was centrifuged twice, or more, at 15,000 rpm for ten minutes till a clear supernatant was obtained. This clear supernatant was further lyophilized on a Labconco freeze dryer and stored at -4^0 C for further use as crude toxin.

3.3.3: Partial Purification

The procedure for partial purification of the crude toxin was based on the method of Shiomi *et al.* (1987) as follows:

i) Preparation of DEAE Cellulose Column: 50 g of DEAE cellulose was swelled in 1.25 liter of distilled water for three hours, and the supernatant was discarded. The sediment was stirred with 1 liter of 1M NaOH for 30 minutes with gentle stirring and allowed to stand for 30 minutes. The supernatant was discarded and the sediment stirred with 1 liter of 1M NaCl for 30 minutes and the supernatant was discarded again. The sediment obtained was again stirred gently with 1 liter of 1M HCL for 30 minutes, soaked with 0.02 M EDTA for 30 minutes and washed with distilled water, and again with Tris- HCl buffer till pH 7.4.

ii) Packing of Column: A properly cleaned glass column (55 x 2.75 cm) was mounted vertically onto a stand and filled to about 15 cm from the bottom with Tris-HCL buffer (pH 7.4) and the outlet was closed. Care was taken to remove air bubbles from below the sintered glass disc and from the flow-regulating device. The slurry of DEAE-Cellulose in Tris-HCl buffer was poured into the column and was allowed to settle under gravity until about 5 cm of cellulose was packed. The outlet was opened to give a moderate flow rate and the process repeated till the end height of packed material reached the

required level of 50 cm. The flow rate was adjusted to 45 ml/hour and washing with the Tris-HCl buffer was continued until the pH of the effluent was the same as that of the buffer.

iii) Sample Application and Elution: Lyophilized crude mucus extract was dissolved in PBS @ 5mg/ml and was dialyzed thoroughly against 0.01 M Tris-HCl buffer (pH 7.4). The level of buffer in column was allowed to run down to the level of packed DEAE cellulose and the outlet was closed. A circular disc of filter paper was placed at the top of the cellulose layer. 5 ml of sample was applied carefully on the bed of the column and the outlet opened till the entire sample had entered the bed.

5 unadsorbed protein fractions of crude toxin were eluted with Tris HCL Buffer and then 10 adsorbed fractions were eluted with a linear gradient of 0.1-1.0 M NaCl in 0.01 M Tris-HCl buffer. Thus a total of 15 fractions (5 unabsorbed and 10 absorbed), each of 15 ml were collected and stored at -4^{0}C for further use.

3.3.4: Estimation of Protein

The concentraiton of protein in the crude extract was estimated by the method of Peterson (1977), using bovine serum albumin (BSA) as standard, reading absorbance spectrophotometrically at 750nm; in case of the fractions, absorbence was read spectrophotometrically at 280 nm.

3.3.5: Mice Bioassay

Male albino mice (Kasauli strain) of 20 ± 2 g weight were procured from M/s Haffkine Biopharma Ltd. Mumbai and were maintained in standard animal

house conditions at CIFE. Mice were provided water and pelleted diet *ad libitum* following all the ethical standards.

Lyophilized crude toxin was dissolved in PBS @ 3mg/ml and injected i.p. into mice, with varying doses of 0.25 ml, 0.50 ml, 0.75 ml and 1.0 ml, whereas only 1.0 ml was injected in case of each fraction. For each dose, triplicate sets of mice were used. The time of injection, activities of injected mice, and time taken for death were recorded and finally mean death time was calculated. The control in each case received an appropriately equal volume of PBS (pH 7.2) in the same manner.

Convulsion, vigorous jumping, rolling over, paresis, disrupted respiratory activity, excessive urination and defecation, apathy, palpitation, and gasping were considered as indicative of toxicity. Mice bioassay was conducted every month, to find out the highest toxicity level in the crude mucus extract.

3.3.6: Calculation of LD_{50}

Assay for LD_{50} involved only one month's sample, the one which showed the highest level of toxicity based on earlier mice bioassays *viz.* November for *A. dussumieri* and September for *O. militaris*. For estimation of 24 hr LD_{50}, triplicate sets of six mice each were injected i.p. with the crude mucus extract in varying doses of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml. LD_{50} was calculated following probit analysis (Finney, 1978).

3.3.7: Assay for Hemolytic Activity

i) Preparation of Erythrocyte Suspension: Hemolytic activity of crude as well as fractionated mucus extract was tested on chicken blood obtained from

nearby slaughter house, using EDTA solution (2.7 g in 100 ml of distilled water) as an anti coagulant @ 5% of the volume of blood, and brought to the laboratory and stored at 4^0 C.

The blood was centrifuged at 5,000 rpm for 7 minutes with PBS (pH 7.2). The supernatant was discarded and the RBC pack was resuspended in saline. This process was repeated thrice and finally RBC concentrate was obtained. This RBC concentrate was mixed with twice its volume of sterilized Alsever's solution (Annexure 1) and stored in the refrigerator for further use, within a period up to 14 days.

ii) Hemolytic Assay by Microtiter Plate Method: A 1% RBC suspension was prepared by diluting RBC with PBS, and used for hemolytic test. Hemolytic assay was performed following the method of Prasad and Venkateshvaran (1997) in "V" shaped Laxbro microtitre plates. Serial two - fold dilution of the mucus (100 µl) were made in PBS (pH 7.2) starting from first well to last well with proper mixing. An equal volume of 1% RBC suspension was added to each well. The plate was gently shaken and allowed to stand for two hours at room temperature and the result was recorded. Appropriate controls were also included in the test. Reciprocal of the highest dilution of the mucus showing the hemolysis was taken as one hemolytic unit (HU). Formation of a compact button formation at the bottom of the well indicated negative hemolytic activity.

3.3.8: Assay for Hemagglutination Activity

Assay for hemagglutination activity was done following Liang and Pan (1995). Procedure for the preparation of erythrocyte was the same to that for

the hemolytic activity. Hemagglutination assays were performed in 'U' bottom microtiter plate using serial dilution of 25 µl of sample (crude/fractionated mucus extract) in physiological saline (0.9% NaCl pH 7.5). Two fold dilution was maintained from first to the last well, and finally a 2% RBC suspension was distributed in each well. Then the plates were shaken for one minute and incubated for one hour at 4^0 C, after which the hemagglutinating activity was recorded. The last dilution of sample showing complete agglutination was taken to contain 1 HA unit.

3.3.9: Edema Forming Activity

The edema forming activity of mucus extracts and fractions was assayed according to the method of Yamakawa *et al.* (1976). Group of five mice were injected with 25 µl of the mucus toxin in the right footpad and with 25 µl of normal saline in the left footpad. After 2 hours, both feet were cut off from the mice sacrificed by chloroform inhalation. Edema ratio was expressed as the percentage increase in weight of the envenomated foot relative to the saline injected foot. The minimum dose was defined as the dose causing 105% edema ratio (ER).

3.3.10:Blocking Test on Edema Forming Activity

The anti histamine drug used was Avil (pheniramine maleate, M/s. Hoechst, Mumbai) while the antiprostaglandin used was Dolonex (anti-prostaglandin analgesic, Pfizer/Dumex, Mumbai) and Atropine (atropine sulphate, Patriot Pharma, Mumbai, Mydriatic agent). These drugs were procured locally and injected i.p. into mice (20 ± 2g) @ 7 mg/kg body weight.

After one hour, as described above under Section 3.3.9, 25 µl of toxins and normal saline were injected into respective feet which were cut off after two hours from sacrificed mice and tested for edema formation. Applying paired t Test at 1% and 5% level, significance of the results was tested.

3.3.11:Antibacterial Activity

The antibacterial activity of the crude as well as fractionated mucus extract of *A. dussumieri* and *O. militaris* was performed by paper disc method following Iguchi *et al.* (1982). One species of gram positive bacteria, *Staphylococcus aureus* and four species of gram negative bacteria, *Aeromonas hydrophila, Escherichia coli, Pseudomonas putrifaciens* and *Vibrio alginolyticus* were drawn from the stock cultures maintained in the Fish Processing and Fish Pathology Laboratories of CIFE. Nutrient broth (Annexure 2) was used for growing the bacteria and antibiotic assay agar (Annexure 3) was used to prepare bacterial lawn.

Bacterial lawns were prepared by pouring 5 ml of overnight grown bacterial culture in nutrient broth in the petri dish containing antibiotic assay agar, allowed to cover the surface and finally the paper disc (8 mm dia; Hi Media, Mumbai) soaked in different concentrations, *viz.* 0.25%, 0.5%, 0.75% and 1.0% of lyophilized skin extract dissolved in PBS were placed at the centre of each plate. After 24 hours incubation at 37^0 C, petri dishes were studied for growth inhibition by sample. A control set was also maintained, but the paper discs used were without mucus extract, having been soaked only in PBS.

3.3.12:Histopathological Study

Kidney, liver, heart and lung were excised from the mice that died upon injection of toxin and was fixed in 10% formalin, for overnight, and then washed thoroughly to remove the fixative. The tissues were dehydrated in 50%, 70% and 90% alcohol for one hour and three times in 100% alcohol for 45 minutes separately. The samples were then cleared in xylene for 2 hours, and embedded in paraffin thrice, each time for 45 minutes. The tissues were then blocked, allowed to cool, cut on a rotary microtome at 7 µm and mounted.

Sections were dewaxed in xylene and dehydrated serially in alcohol and were then stained in Delafield's hamatoxylene for 7 minutes. Stained sections were washed in tap water, dipped in 2% acid alcohol, washed again in tap water followed by Scott's tap water substitute then washed in distilled water. Washed sections were stained in eosin for 5 minutes. The sections were then washed in water and dehydrated through graded series of alcohol for 1 minute each. The stained sections were finally cleaned in xylene and mounted on glass slides with DPX. Prepared sections were examined and photographed under an Leika microscope.

3.3.13:Histological Study of Skin and Spines

The pectoral spines of both the fish were fixed in Bouin's fluid for 24 hours and decalcified for 4 weeks in 70% alcohol with 3% hydrochloric acid following Bhimachar (1944). Permanent sections of decalcified spine and skin were prepared as above (Section 3.3.12) and photographed under microscope.

3.4: Results

3.4.1: Extraction of Crude Toxin

The quantity of mucus that was scrapped from 12 fishes in a given month varied in quantity between 7 and 12 ml depending on the size of the fish. Smaller fishes were observed to yield more quantity of mucus. Upon lyophilization, this quantity of mucus yielded 300mg to 500mg of crude toxin in powdery form.

3.4.2: Estimation of Protein

The highest protein content in the crude toxin was 363 µg/mg (November 1997) in the case of *A. dussumieri* and 225 µg/mg (September 1997) in the case of *O. militaris* whereas the least quantity of protein was 275 µg/mg (*A. dussumieri*) and 190 µg/mg (*O. militaris*), both in February 1998. The month-wise data on protein content of the crude toxin is presented in Table 5 and Fig. 4 while that of the fractions in Table 6 and Fig. 5.

3.4.3: Mice Bioassay involving Crude Toxin

Crude mucus extract of both the species exhibited toxicity when tested on mice. The results of the bioassay involving crude toxin are presented in Tables 7 and 8. The mice injected with the mucus extracts of both the fishes, *A. dussumieri* and *O. militaris* exhibited the following symptoms: lethargy, apathy, excessive defaecation and urination, paresis of hindlimbs, convulsions, jumping tendency, etc.

The crude mucus extract of *A. dussumieri* showed the highest toxicity in November 1997 at a dose of 0.25 ml and killed the mice in 50 minutes. In the

case of *O. militaris* the most toxic dose was 0.50 ml, in September 1997,with a death time of 80 minutes.

Crude toxin of *A. dussumieri* exhibited such toxicity throughout the year whereas that *O. militaris* was not lethal to mice during February and March. Considerable monthwise variations in the level of toxicity were, however discernible in both the cases. Thus, in case of *A. dussumieri*, crude toxin of January, February, March and June was lethal to mice at a dose of 1.0 ml (corresponding to 0.825 to 0.945 mg protein) but in November a dose of 0.25 ml, corresponding to 0.272 mg protein) was lethal.

Similarly, in the case of crude toxin of *O. militaris* the lethal dose was 1.0 ml (corresponding to 0.600 to 0.660 mg protein) during the periods May to August and October to December. A dose of 0.50ml (=0.337mg protein) was lethal to mice in September.

LD_{50} values of the crude mucus toxin were estimated to be 20.06 mg/kg and 25.86 mg/kg for *A. dussumieri* and *O. militaris* respectively. The results are presented in Tables 9 and 10.

3.4.3: Mice Bioassay involving Partially Purified Toxin

Toxicity of partially purified mucus extracts of *A. dussumieri* upon i. p. injection to the mice revealed three lethal factors, one (U3) in unadsorbed and two (F5 and F8) in adsorbed fractions. Fraction F2, F4, F6, and F7 were not lethal but elicited the symptoms of toxicity; mice injected with these fractions recovered fully after a period of time that varied between 4 Hrs 25 min and 30 Hrs. Results of these bioassays are presented in Tables 11 and 12.

In the case of *O. militaris,* Fractions U4, F7, and F10 were lethal to mice upon i. p. injection, death time being 65 min, 42 min, and 14 min respectively. Fractions U1 and F6 were not lethal but showed the symptoms of toxicity. Recovery time varied between 12 Hr 10 min and 14 Hr 40 min.

3.4.4: Hemolytic Assay

Hemolytic assay conducted against chicken erythrocytes showed that the crude mucus extracts as well as partially purified fractions of both fishes to be hemolytic.

Crude toxin of *A. dussumieri* exhibited hemolytic activity of 16 HU in August, September, October, November, and December while that of *O. militaris* exhibited the highest level of 8 HU in May, September and October. Results of hemolytic activity of the crude mucus extracts are presented in Table 13 and Plate 2.

Partially purified fractions of *A. dussumieri* had varying hemolytic activity from 4 HU to 32 HU during the study period; monthwise, F6 fraction of April, U4 fraction of May, and F8 fraction of November showed the highest activity of 32 HU. The lowest hemolytic activity *viz.* 4HU was observed in September (F7), December (F3), January (F3 and F8), February (U2, F6) and March 1998 (U2). The results are presented in Table 14 and Plate 3.

Partially purified fractions of *O. militaris* showed the highest hemolytic activity of 16 HU during May (F6) and September (F6 and F10). The results are presented in Table 15 and Plate 4.

3.4.5: Hemagglutination Assay

The crude mucus extracts of both fishes were found to have partial hemagglutinating activity that became more prominent upon partial purification. In the case of *A. dussumieri,* the highest hemagglutinating titre of 32 HAU was observed in November (F9). However, partially purified fractions of *O. militaris* were found to have the highest hemagglutinating titre of 32 HAU in September (F8 and F9). Results of the assays for hemagglutinating activity are described in Tables 14 and 15, and Plates 5 and 6.

3.4.6: Assay of Edematic Activity

The results on edematic activity are presented in Tables 16 and 17 and Figs. 6 and 7. Crude as well as partially purified fractions of both the fish exhibited edematic activity. Crude mucus extracts of *A. dussumieri* was more edematic with an edematic activity of 154.29%, whereas that of *O. militaris* had an edematic activity of 137.9%. Only two partially purified fractions (F5 and F7) of *A. dussumieri* showed edema forming activity. Partially purified mucus extract of *O. militaris* also had two edematic factors, one in Unadsorbed (U1) and another in adsorbed (F2) fractions.

3.4.7: Blocking of Edematic Activity

Blocking of edematic activity with three different drugs, Avil, Dolonex and Atropine had different effects. Dolonex was found to be more effective in bringing down edema level than Avil. However, both of them were found to be statistically insignificant at 1% and 5% level. Atropine in all cases was found to

increase edema level, but this increase was also statistically insignificant. The results are presented in Tables 16 and 17.

3.4.8: Anticabterial Assay

Assays for antibacterial activity involving crude toxin as well as partially purified fractions from both the fishes, in all the concentrations used, yielded negative results with reference to the gram positive *(S. aureus)* and gram negative (*A. hydrophila, E. coli, P. putrifaciens* and *V. alginolyticus)* bacteria.

3.4.9: Gross Anatomical Changes

Autopsy of mice dead upon envenomation revealed dark discoloration of the liver whereas the kidney and heart did not show any discoloration or other changes. Blood clots in the anterior body cavity as also the lungs were clearly visible to the naked eye.

3.4.10: Histopathology

Histopathological sections revealed thickening of the alveolar walls as also increase in the alveolar spaces in the lungs (Plate7). In case of the liver the sinusoids were distended and disrupted with accumulated RBC's inside; pycnotic as also karyoorrhectic nuclei were also observed inaddition to karyolysis (Plate 8). Blood clots were also observed in liver and to some extent in the lungs. Heart and kidney tissues did not show any appreciable damage (Plates 9 and 10).

3.4.11: Histological Studies

Histological sections of the skin spine sheath and revealed no grandular cells in the epidermis (Plates 11 and 12). Spine sections (Plates 13 and 14) revealed mucus aggregations immediately outside the cartilaginous tissue and the presence of adipose tissues and what appear to be glycoproteins inside the lumen of the spine.

3.5: Discussion

Both species of catfishes studied are crinotoxic but the crude mucus extract of *A. dussumieri* had a stronger toxicity than that of *O. militaris*.

The observed symptoms of toxicity compare well with those reported for other catfishes such as *Plotosus lineatus* (Shiomi *et al.* 1987) and *Arius thalassinus* (Al-Hassan *et al.* 1985,1986, 1987). Using rabbit models, Al-Hassan *et al.* (1987) could not detect observable symptoms upto very large doses of 10-20 mg/kg body weight when the gel components of *A. thalassinus* was injected by the subcutaneous or intra peritoneal route. Contrary to this, symptoms of toxicity were observed in mice upon i.p. administration of the crude toxin in the present study, but the doses involved *viz.* 0.600 mg/20g body weight (*O. militaris*) to 0.945 mg/20g body weight (*A. thalassinus*), which correspond to 30 and 47.2 g/kg respectively, are higher. It can thus be deduced that the crude toxin of either species was less active than that of *A. thalassinus.*

Upon partial purification, 3 lethal factors each in case of *A.dussumieri* and *O. militaris* were discernible but the protein levels in the former had been higher than those of the latter. A single such lethal factor was isolated in the

case of *A. bilineatus* by Thomson *et al.* (1998). Shiomi *et al.* (1986) isolated two lethal factors from the mucus of *Plotosus lineatus* and purified one in a later study (Shiomi *et al.* 1987). Mucus of *A. dussumieri* yielded 3 lethal factors and that of *O. militaris* also 3 lethal factors (Variath and Venkateshvaran, 1999) but in their case 2 lethal factors in each species were from the unadsorbed fractions. The present study is at variance from this in that only one lethal factor in each case was from the unadsorbed fractions, despite the fact the chromatographic methods involved wre the same in both studies.

Median death time for these lethal factors was reported by Variath and Venkateshvaran (1999) to vary between 45 seconds and 8 minutes (*A. dussumieri*) and between 1 and 3 minutes (*O. militaris*) but in the present case it varied between 8 and 33 minutes in the former and 14 and 65 minutes in the latter species. The differences observed could be due to the fact that Variath and Venkateshvaran (1999) did not use lyophilized samples but only dialyzed the crude extract before fractionation. It is also possible that lyophilization could have caused a loss of potency, since Russell and Brodie (1974) reported that, on lyophilization, as much as half of the lethal property may sometimes be lost.

LD_{50} values for various crinotoxins are available (1.5mg/kg by Al-Hassan *et al.* 1985; *P. canius*, mucus 7.0µg/g by Othman *et al.* 1993; 0.045 mg/kg of *A. bilineatus* by Thomson *et al.* 1998; 6.2 – 22mg/kg of *Anguilla japonica* by Shiomi *et al.* 1990) but comparisons with the present data are different due to the differences in the route of administration and the methods or levels of purification as also the test organisms used.

Crude as well as partially purified toxins of both the species exhibited a potent hemolytic activity against chick erythrocytes. Upon partial purification, 3 hemolytic factors each in the case of *A. dusumieri* and *O. militaris* were obtained. Hemolytic activity has been shown to be present in the mucus of various catfishes such as *A. thalassinus* (Al-Hassan *et al.* 1982; 1986) *A. bilineatus* (Thomson *et al.* 1998) *P. lineatus* (Al-Lahham *et al.* 1987 and Shiomi *et al.* 1986) *P. canius* (Othman *et al.* 1993), eels such as *Anguilla japonica* (Swzuki, 1985) *Lycodontis nudivomer* (Randall *et al.* 1981) soapfishes, *Pogonoperca punctata* (Hashimoto and Oshima, 1972; Oshima *et al.* 1974), Moses sole *Pardachirus marmoratus* (Primer and Zoltkin, 1975; Lazarovici *et al.* 1986) etc.

Hemaggluting activity, on the other hand, was also present, in addition to hemolytic activity, to some extent in the crude toxin. However, after partial purification, the hemagglutinating activity was more pronounced. Al-Hassan *et al.* (1986) isolated and purified a lectin from the epidermal secretion of *A. thalassinus* which comprised about 2% of the total gel protein, lacked carbohydrates and contained no unusual types or amounts of aminoacids; the lectin was reported to agglutinate a wide range or red blood cell types. Hemagglutinating activity was also reported from the skin mucus of the Moray eel, *Lycodontis nudivomer* by Randall *et al.* (1981), and that of the Japanese eel *Anguilla japonica* by Sujuki (1985).

A total of 2 edematic factors were discermible in each of the species studied upon partial purification of the crude toxin. Edema-forming activity, in conformity with the present data, has also been reported in case of mucus

extracts from *P. lineatus* (Shiomi *et al.* 1986, 1987, 1988) as also from seasnake venom (Tu, 1988).

It is noteworthy that, in the present investigation all lethal factors also had either hemolytic, or edematic activities; also, there were fractions with only hemolytic or only edemtic activities without concomitant lethal activity. Such combined lethal and edematic activities of the same fraction have been reported in the case of *P. lineatus* by Shiomi *et al.* (1986, 1987, 1988) whereas reports of combined lethal and hemolytic activities in the same fractions are very common (Othman *et al.* 1993 in *P. canius*; Al-Hassan *et al.* 1985, 1986, 1987 in *A. thalassinus*). Randall *et al.* (1981) also observed that the fish and mice toxic activities in the crinotoxin of moray eel behaved parallel to the hemolysin and, therefore, suggested that the hemolytic and toxic activities belonged to the same component. Although the hemolytic components are often elaborated from speacialized venom glands, they are sometimes present in skin secretions also (Cameron and Endean, 1973).

The present study also revealed certain factors that had only one of the activities, either lethal or hemolytic and such results are corroborated by the findings of Al-Lahham *et al.* (1987) in case of A. thalassinus where the hemolytic factor was not ichthyotoxic nor toxic to rabbits upon i.v. injection.

Autopsy revealed impact of the toxin on liver and lungs during the present study that were supported by the histopathological data. Al-Hassan *et al.* (1985) reported the skin toxin of *A. thalassinus* produced a time dependent toxic effect on the liver and heart of rabbits, although the heart tissue was not affected by the toxins in the present study. Skin toxin of *A. bilineatus* affected the lungs and liver of treated rabbits (Alnaqueeb *et al.* 1989) as had also been

observed in the current investigation; heart and kidney were found to be unaffected in their study but slight damage to the kidney was discernible in the present study.

Mucus appears to be produced by the epidermal cells since, contrary to all earlier findings, no glandular cells were discernible in the skin sections of the fishes. The presence of the glycoprotein-like material in the lumen of the spine is also not reported earlier and is worth investigating further; this, together with the mucus aggregation might have a role in the toxicity of the spine, an aspect not addressed by the present study.

Absence of antibacterial activity in the mucus of both the species is in conformity with earlier findings of Al-Hassan *et al.* (1987) in case of *A. thalassinus,* the only catfish in which this aspect has been addressed to. Variath and Venkateshvaran (1999) also did not find antibacterial activity in the same two species *viz., A. thalassinus* and *O. militaris.*

Table 5

Showing protein levels in crude mucus of *Arius dussuumieri* and *Osteogeneiosis militaris* during the study period

Month	Protein level (µg/ mg)	
	A. dussuumieri	***O. militaris***
March, 1997	305	195
April, 1997	315	220
May, 1997	340	214
June, 1997	315	200
July, 1997	350	205
August, 1997	353	220
September, 1997	340	225
October, 1997	352	220
November, 1997	363	207
December, 1997	358	200
January, 1998	310	210
February, 1998	275	190
March, 1998	300	200

Table 6

Showing the Absorbance of fractions at 280 nm

S.No	*Arius dussuumieri* (November, 97)	*Osteogeneiosus militaris* (September, 97)
1	0.1371	0.0997
2	0.1413	0.0893
3	0.1892	0.0973
4	0.1661	0.1043
5	0.1534	0.0873
6	0.1692	0.0871
7	0.1743	0.0828
8	0.1645	0.0772
9	0.1828	0.0810
10	0.2026	0.0865
11	0.1725	0.0922
12	0.1866	0.1424
13	0.2558	0.5482
14	0.5349	0.3647
15	0.3758	0.2311

Table 7. Showing toxicity of crude mucus extracts from *Arius dussumieri* to male albino mice (20 ± 2 g)

	Mar, 97	Apr, 97	May, 97	Jun, 97	Jul, 97	Aug, 97	Sep, 97	Oct, 97	Nov, 97	Dec, 97	Jan, 98	Feb, 98	Mar, 98
Protein Conc. (mg/ml)	0.915	0.945	1.02	0.945	1.050	1.059	1.020	1.056	1.089	1.074	0.930	0.825	0.910
Dose 0.25 ml.	NL	NL	NL	NL	NL	NL	NL	NL	L	NL	NL	NL	NL
MDT(Hrs.)									0.50				
Dose 0.50 ml.	NL	NL	NL	NL	L	NL	NL	L	*	L	NL	NL	NL
MDT(Hrs.)					7.40			8.15		2.40			
Dose 0.75 ml.	NL	L	L	NL	*	L	L	*	*	*	NL	NL	NL
MDT(Hrs.)		8.40	4.10			2.13	7.36						
Dose 1.0 ml.	L	*	*	L	*	*	*	*	*	*	L	L	L
MDT(Hrs.)	3.10			2.25							4.50	6.15	4.20
Control 1.0 ml.	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL

NL: NOT LETHAL L: LETHAL * : NOT TESTED MDT: MEAN DEATH TIME

Table 8. Showing toxicity of crude mucus extracts from *Osteogeneiosus militaris* to male albino mice (20 ± 2 g)

	Mar, 97	Apr, 97	May,97	Jun, 97	Jul, 97	Aug, 97	Sep, 97	Oct, 97	Nov, 97	Dec, 97	Jan, 98	Feb, 98	Mar, 98
Protein Conc. (mg/ml)	0.585	0.660	0.660	0.642	0.600	0.615	0.675	0.660	0.621	0.600	0.630	0.570	0.580
Dose 0.25 ml.	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
MDT(Hrs.)													
Dose 0.50 ml.	NL	NL	NL	NL	NL	NL	L	NL	NL	NL	NL	NL	NL
MDT(Hrs.)							1.20						
Dose 0.75 ml.	NL	L	NL	NL	NL	NL	*	L	NL	NL	NL	NL	NL
MDT(Hrs.)		4.30						4.30					
Dose 1.0 ml.	NL	*	L	L	L	L	*	*	L	L	L	NL	NL
MDT(Hrs.)			6.10	7.30	10.15	17.00			20.15	23.00	19.30		
Control 1.0 ml.	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL

NL: NOT LETHAL L: LETHAL * : NOT TESTED MDT: MEAN DEATH TIME

Table 9. Showing LD_{50} (24 Hrs.) of crude mucus extracts (Nov. 97) from *Arius dussumieri* injected i.p. into male albino mice (20 ± 2 g.)

Dose (ml)		0.2	0.4	0.6	0.8	1.0
Conc. of protein (mg/kg)		11	22	33	44	55
Treatment						
R1	No. of mice	6	6	6	6	6
	No. of death	0	3	4	5	6
	Death %	0	50	66.66	83.33	100
R2	No. of mice	6	6	6	6	6
	No. of death	1	3	4	5	6
	Death %	16.6	50	66.66	83.33	100
R3	No. of mice	6	6	6	6	6
	No. of death	2	4	5	4	5
	Death %	33.33	66.66	83.33	66.66	83.33

Table 10. Showing LD_{50} (24 Hrs.) of crude mucus extracts (September, 97) from *Osteogeneiosus militaris* injected i.p. into male albino mice (20 ± 2 g.)

Dose		0.2 ml	0.4 ml	0.6 ml	0.8 ml	1.0 ml
Protein Conc. (mg/kg)		6.50	13.50	20.00	27.00	33.75
Treatment	No. of mice	6	6	6	6	6
R1	No. of death	0	0	1	3	4
	Death %	0	0	16.66	50	66.66
R2	No. of mice	6	6	6	6	6
	No. of death	0	0	2	3	4
	Death %	0	0	33.33	50	66.66
R3	No. of mice	6	6	6	6	6
	No. of death	0	1	2	4	3
	Death %	0	16.66	33.33	66.66	50

Table 11

Showing the toxicity of partially purified mucus toxin of *A. dussumieri* to male albino mice (20 ± 2g) @ 1ml/mice i.p.

S.No	Fractions	Protein in mg	Mean Death Time	Remarks
1	U1	0.137	-	Not lethal
2	U2	0.141	-	Not lethal
3	U3	0.189	33 Min.	Lethal
4	U4	0.166	-	Not lethal
5	U5	0.153	-	Not lethal
6	0.1M	0.169	-	Not lethal
7	0.2M	0.174	-	Not lethal, Recovered after 4 Hrs 25 min.
8	0.3M	0.164	-	Not lethal
9	0.4M	0.182	-	Not lethal, Recovered after 20 Hrs.
10	0.5M	0.202	18 Min.	Lethal
11	0.6M	0.172	-	Not lethal, Recovered after 10 Hrs.
12	0.7M	0.186	-	Not lethal, Recovered after 30 Hrs.
13	0.8M	0.255	8 Min.	Lethal
14	0.9M	0.534	-	Not lethal
15	1.0M	0.375	-	Not lethal

Table 12

Showing the toxicity of partially purified mucus toxin of *O. militaris* to male albino mice (20 ± 2g) @ 1ml/mice i.p.

S.No	Fractions	Protein in mg.	Mean Death Time	Remarks
1	U1	0.099	-	Not lethal, Recovered after 14 Hrs 40 min.
2	U2	0.089	-	Not lethal
3	U3	0.097	-	Not lethal
4	U4	0.104	1.05 Hrs.	Lethal
5	U5	0.087	-	Not lethal
6	0.1M	0.087	-	Not lethal
7	0.2M	0.082	-	Not lethal
8	0.3M	0.077	-	Not lethal
9	0.4M	0.081	-	Not lethal
10	0.5M	0.086	-	Not lethal
11	0.6M	0.092	-	Not lethal, Recovered after12 Hrs10 Min.
12	0.7M	0.142	42 Min.	Lethal
13	0.8M	0.548	-	Not lethal
14	0.9M	0.364	-	Not lethal
15	1.0M	0.231	14 Min.	Lethal

Table13. Showing hemolytic activity of crude mucus extracts of *A. dussumieri* and *O. militaris* on chicken blood

Month	*A. dussumieri*		*O. militaris*	
	Hemolytic Unit (HU)	HU/mg Protein	Hemolytic Unit (HU)	HU/mg Protein
Mar, 97	4	160/0.915	2	80/0.585
April, 97	4	160/0.945	4	160/0.660
May, 97	8	320/1.02	8	320/0.660
June, 97	8	320/0.945	4	160/0.642
July, 97	8	320/1.050	4	80/0.600
August, 97	16	640/1.059	2	80/0.615
September, 97	16	640/1.020	8	320/0.675
October, 97	16	640/1.056	8	320/.660
November, 97	16	640/1.08	4	160/0.621
December, 97	16	640/1.074	2	80/0.600
Jan, 98	8	320/0.930	4	160/0.630
Feb, 98	4	160/0.825	2	80/0.570
March, 98	4	160/0.910	2	80/0.580

Table 14. Showing hemolytic and hemagglutinating activities in the fractions of *A. dussumieri* on chicken blood

Months	Fractions														
	U1	U2	U3	U4	U5	0.1M	0.2M	0.3M	0.4M	0.5M	0.6M	0.7M	0.8M	0.9M	1.0M
Mar, 97	-	8HU	-	-	-	-		-	16HU	-	-	-	16HU	16HAU	-
April, 97	-	-	16HU	-	-	-	-	8HU	-	-	32HU	-	-	8HAU	-
May, 97	-	-	-	32HU	-	-	16HU	8HU	-	-	-	8HU	-	-	8HAU
June, 97	-	-	8HU	-	-	-	-	4HU	-	-	-	16HU	-	16HAU	-
July, 97	-	-	-	16HU	-	-	-	8HU	-	-	-	-	16HU	16HAU	-
August, 97	-	-	16HU	-	-	-	8HU	-	-	-	-	-	16HU	-	4HAU
September, 97	-	-	-	8HU	-	-	-	8HU	-	-	-	4HU	-	8HAU	-
October, 97	-	-	-	8HU	-	-	-	16HU	-	-		-	8HU	4HAU	8HAU
November, 97	-	-	16HU	-	-	-	8HU	-	-	-	-	-	32HU	32HAU	8HAU
December,97	-	-	16HU	-	-	-	4HU	-	-	-	-	16HU	-	16HAU	8HAU
Jan, 98	-	8HU	-	-	-	-	-	4H	-	-	-	-	4HU	-	4HAU
Feb, 98	-	4HU	-	-	-	-	-	8HU	-	-	4HU	-	-	-	4HAU
March, 98	-	4HU	-	-	-	-	-	-	8HU	-	-	-	8HU	4HAU	-

HU: Hemolytic Unit

HAU : Hemagglutinating Unit

U1 – U5 : Unadsorbed fractions

0.1 – 1.0 M : Adsorbed fractions (F1-F10)

Table 15. Showing hemolytic and hemagglutinating activities in the fractions of *O. militaris* on chicken blood

Months	Fractions														
	U1	U2	U3	U4	U5	0.1M	0.2M	0.3M	0.4M	0.5M	0.6M	0.7M	0.8M	0.9M	1.0M
Mar, 97	-	-	-	-	4HU	-	-	-	-	-	8HU	-	-	8HAU	-
April, 97	-	-	-	4HU	-	-	-	-	-	8HU	-	-	4HAU	4HAU	-
May, 97	-	-	-	8HU	-	-	-	-	-	-	16HU	-	4HAU	8HAU	8HU
June, 97	-	-	8HU	-	-	-	-	-	-	4HU	4HU	-	8HAU	-	-
July, 97	-	-	-	4HU	-	-	-	-	-	-	8HU	-	-	8HAU	2HU
August, 97	-	-	-	4HU	-	-	-	-	-	8HU	-	-	4HAU	8HAU	4HU
September, 97	-	-	-	4HU	-	-	-	-	-	-	16HU	-	32HAU	32HAU	16HU
October, 97	-	-	-	8HU	-	-	-	-	-	-	8HU	-	16HAU	8HAU	-
November, 97	-	-	4HU	-	-	-	-	-	-	-	8HU	-	8HAU	4HAU	4HU
December,97	-	-	-	4HU	-	-	-	-	-		4HU	-	-	8HAU	4HU
Jan, 98	-	-	-	8HU	-	-	-	-	-	-	8HU	-	4HAU	8HAU	8HU
Feb, 98	-	-	4HU	-	-	-	-	-	-	8HU	-	-	4HAU	4HAU	4HU
March, 98	-	-	-	-	4HU	-	-	-	-	-	4HU	-	-	4HAU	-

HU: Hemolytic Unit

HAU: Hemagglutinating Unit

U1 – U5: Unadsorbed fractions

0.1 – 1.0 M: Adsorbed fractions(F1-F10)

Table 16. Showing edematic activity of the crude mucus extract and fractions (Nov. 97) of *A. dussumieri* and its blocking

A : Mice i.p. injected with only the toxin extract

B : Mice i.p. injected previously with Avil @ 7mg/kg.

C : Mice i.p. injected previously with Dolonix @ 7mg/kg.

D : Mice i.p. injected previously with Atropine @ 7mg/kg.

Toxin solution	Edema Ratio (%)			
	A	B	C	D
Crude extract	154.29 ± 0.144	149.63 ± 0.227	140.438 ± 0.286	160.726 ± 0.167
Fraction No. 5	145.90 ± 0.523	134.91 ± 0.376	131.52 ± 0.366	145.97 ± 0.400
Fraction No. 7	135.13 ± 0.276	130.56 ± 0.376	125.116 ± 0.231	141.10 ± 1.514

Table 17. Showing edematic activity of the crude mucus extract and fractions (Sep. 97) of *O. militaris* and its blocking

A : Mice i.p. injected with only the toxin extract

B : Mice i.p. injected previously with Avil @ 7mg/kg.

C : Mice i.p. injected previously with Dolonix @ 7mg/kg.

D : Mice i.p. injected previously with Atropine @ 7mg/kg.

Toxin solution	Edema Ratio (%)			
	A	B	C	D
Crude extract	137.9 ± 0.279	131.68 ± 0.460	125.49 ± 0.222	146.79 ± 0.545
Unadsorbed (U1)	135.112 ± 0.178	132.25 ± 0.866	126.90 ± 0.433	139.934 ± 2.365
Fraction No. 7	142.15 ± 0.255	135.62 ± 0.677	127.56 ± 0.541	144.61 ± 1.501

FIG. 3

FUNCTIONS OF FISH MUCUS
(REDRAWN FROM SHEPHARD, 1994)

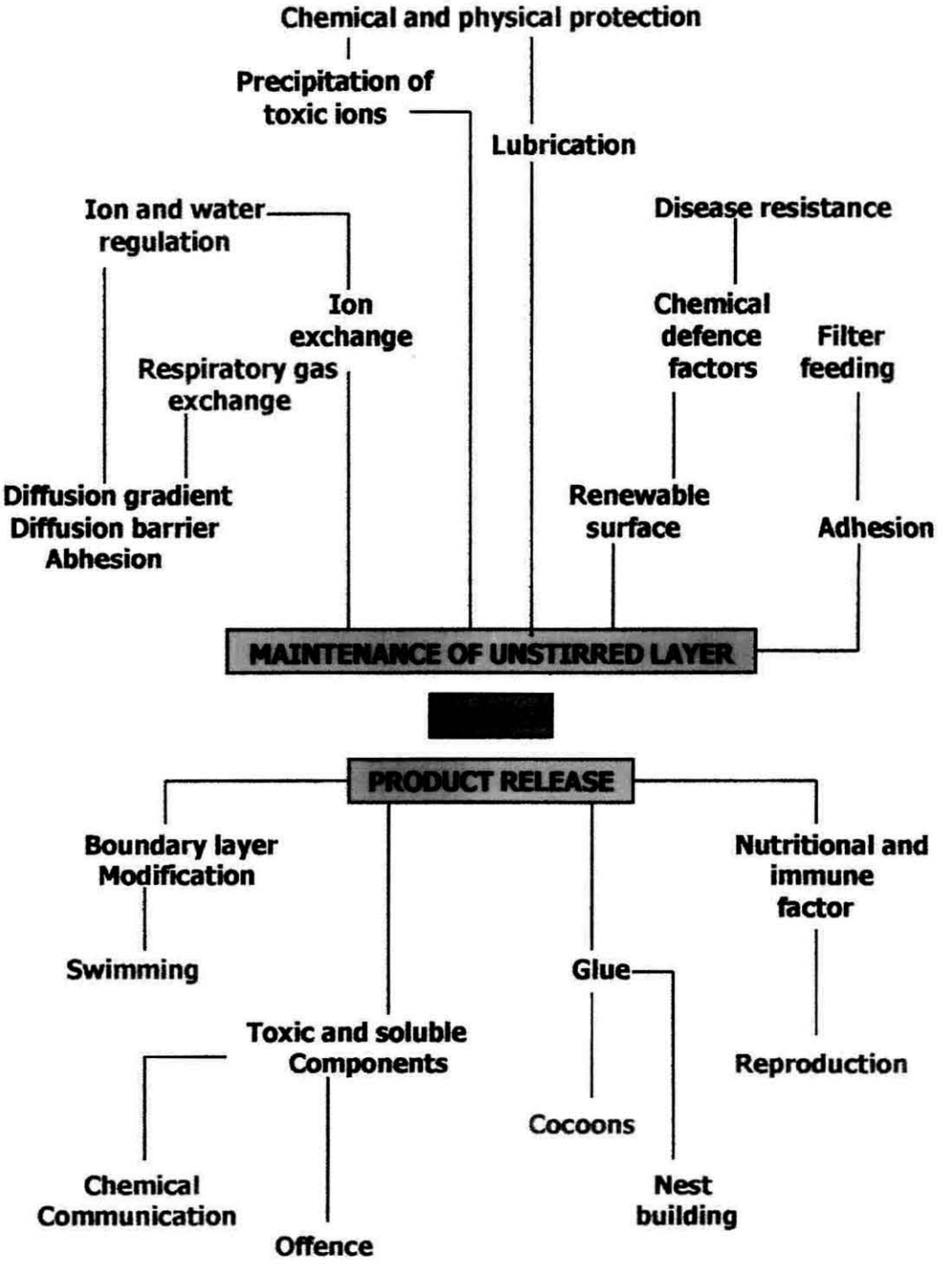


Fig. 4: Protein concentration of crude mucus extracts of *A. dussumieri* and *O. militaris*

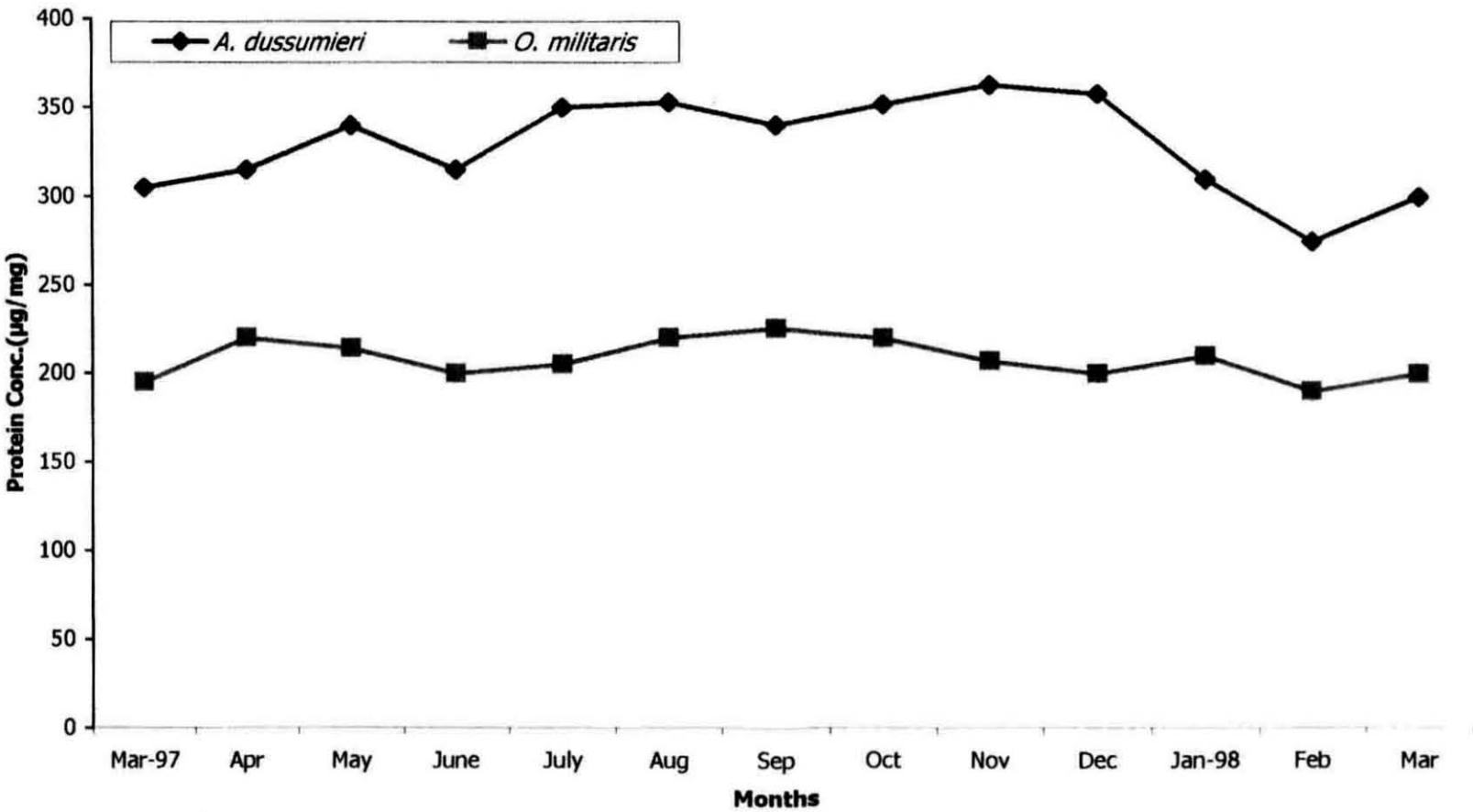


Fig. 5: Absorbance of fractions of *A. dussumieri* (Nov.97) and *O. militaris* (Sep.97) at 280nm

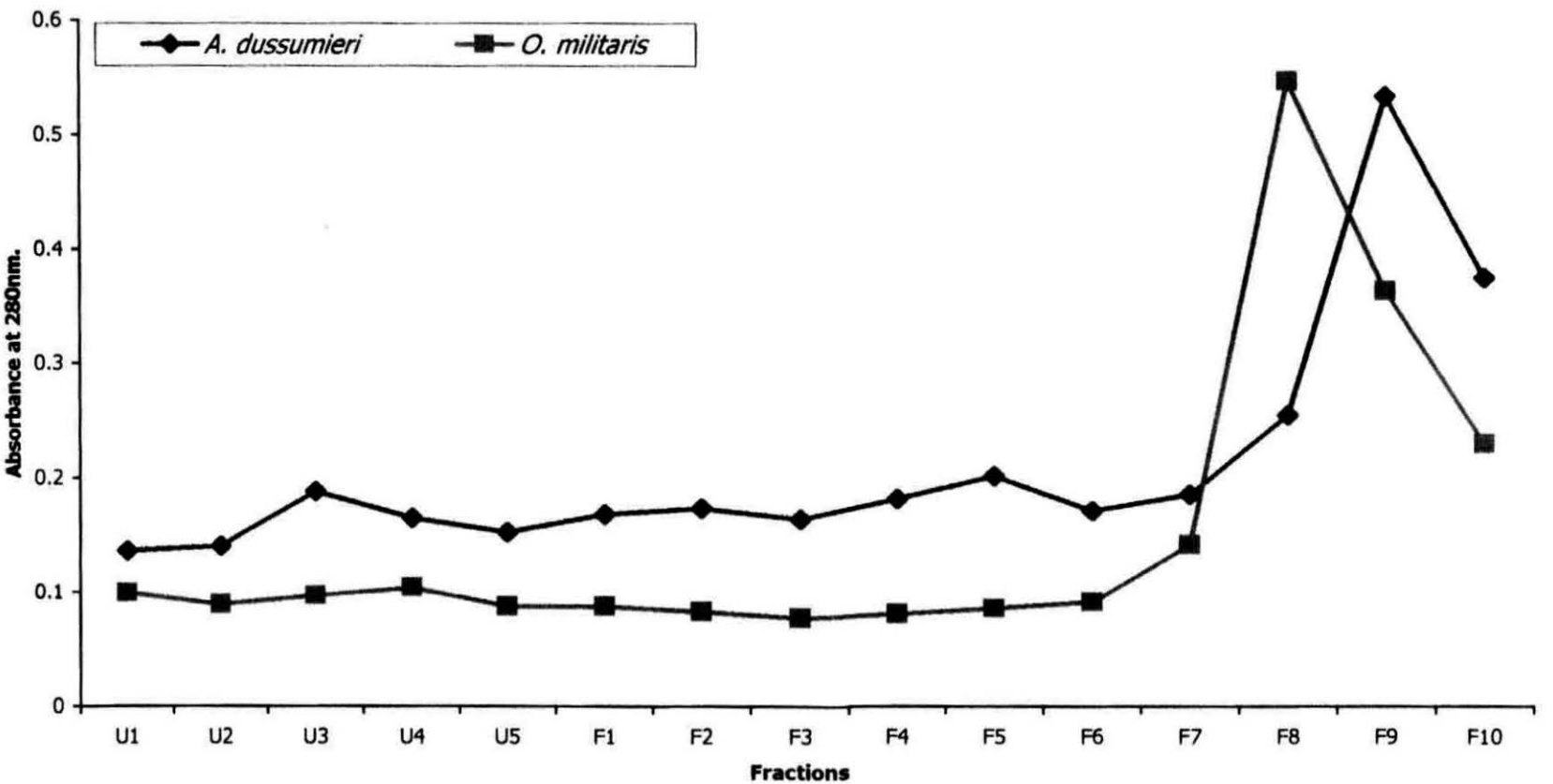
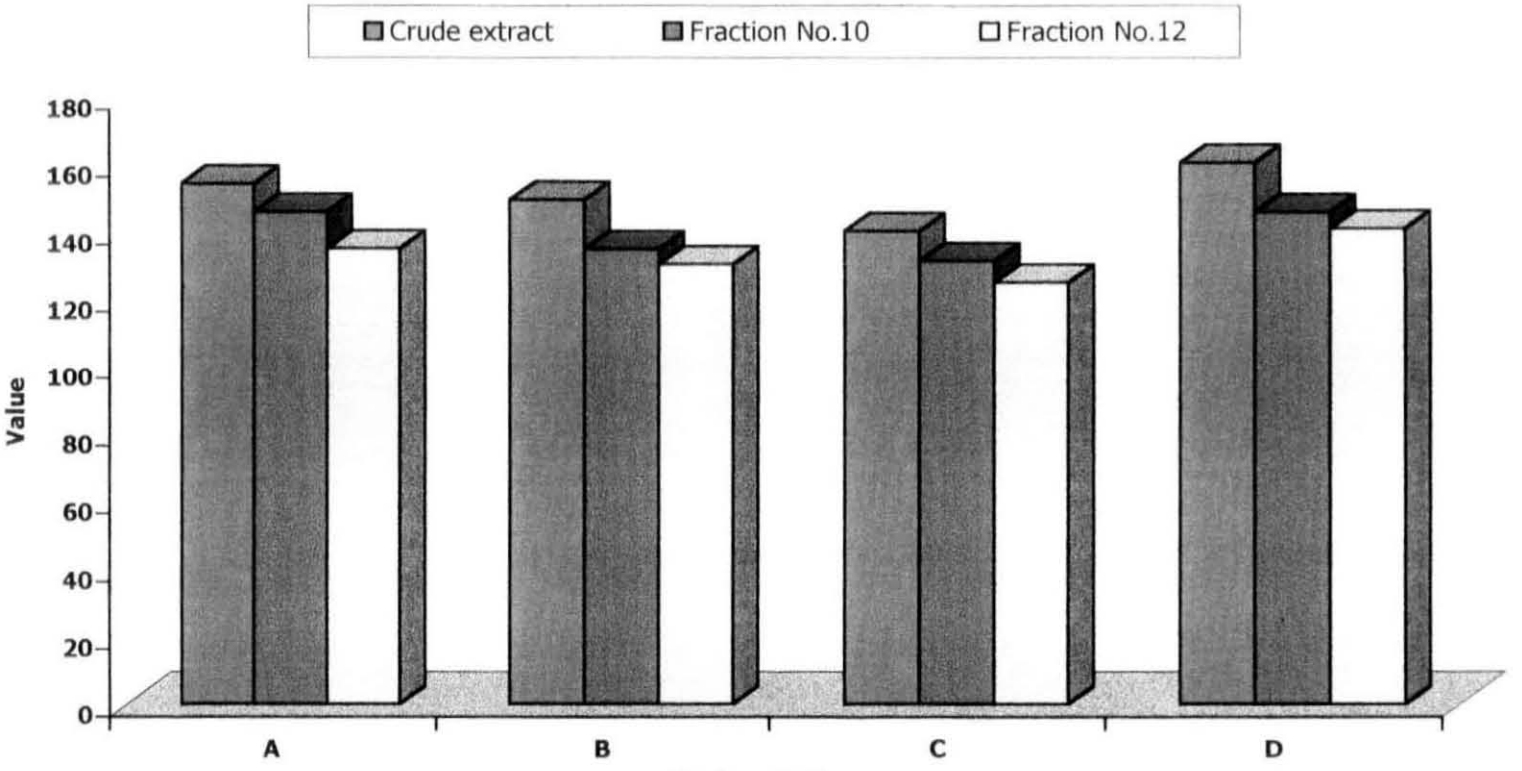
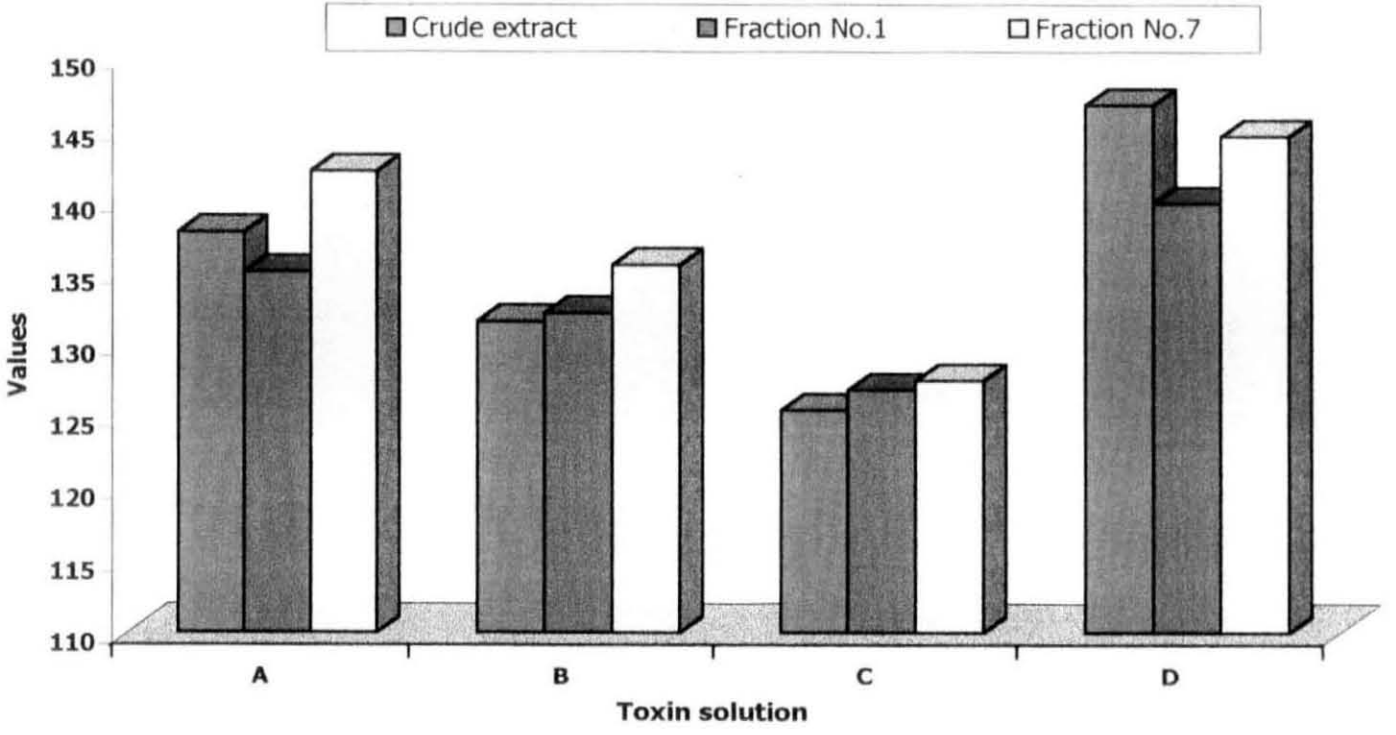


Fig. 6: The Edema-forming activity of the crude mucus extract and fractions (Nov.97) of *A. dussumieri* and its blocking



A: Mice injected with only the toxin extract
B: Mice injected previously with Avil @ 7mg/kg.
C: Mice injected previously with Dolonex @ 7mg/kg.
D: Mice injected previously with Atropine @ 7mg/kg.

Fig. 7: The Edema-forming activity of the crude mucus extract and fractions (Sep.97) of *O. militaris* and its blocking



A: Mice injected with only the toxin extract
B: Mice injected previously with Avil @ 7mg/kg.
C: Mice injected previously with Dolonex @ 7mg/kg.
D: Mice injected previously with Atropine @ 7mg/kg.

Plate 2

Showing hemolytic activity of crude mucus toxins of *A. dussumieri* and *O. militaris* on chicken blood.

Row 1: *A. dussumieri*
Row 2: *O. militaris*
+: Positive Control -:Negative Control

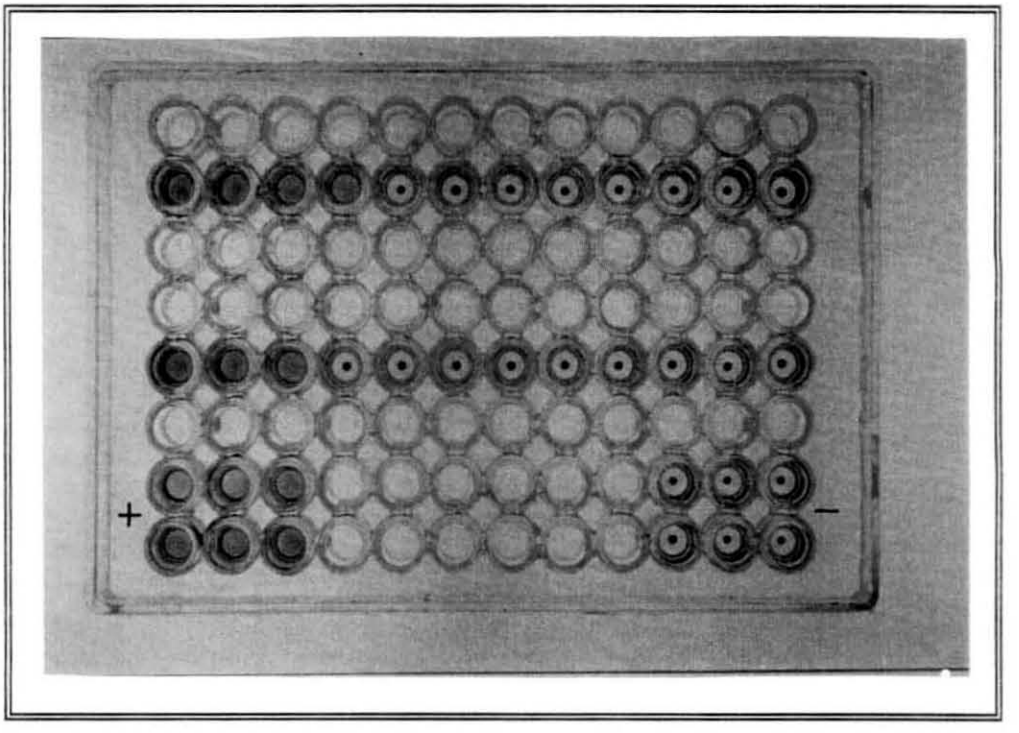


Plate 3

Showing hemolytic activity of partially purified skin toxins of *A. dussumieri* on chicken blood

Row 1: U3
Row 2: F2
Row 3: F8

+: Positive Control -:Negative Control

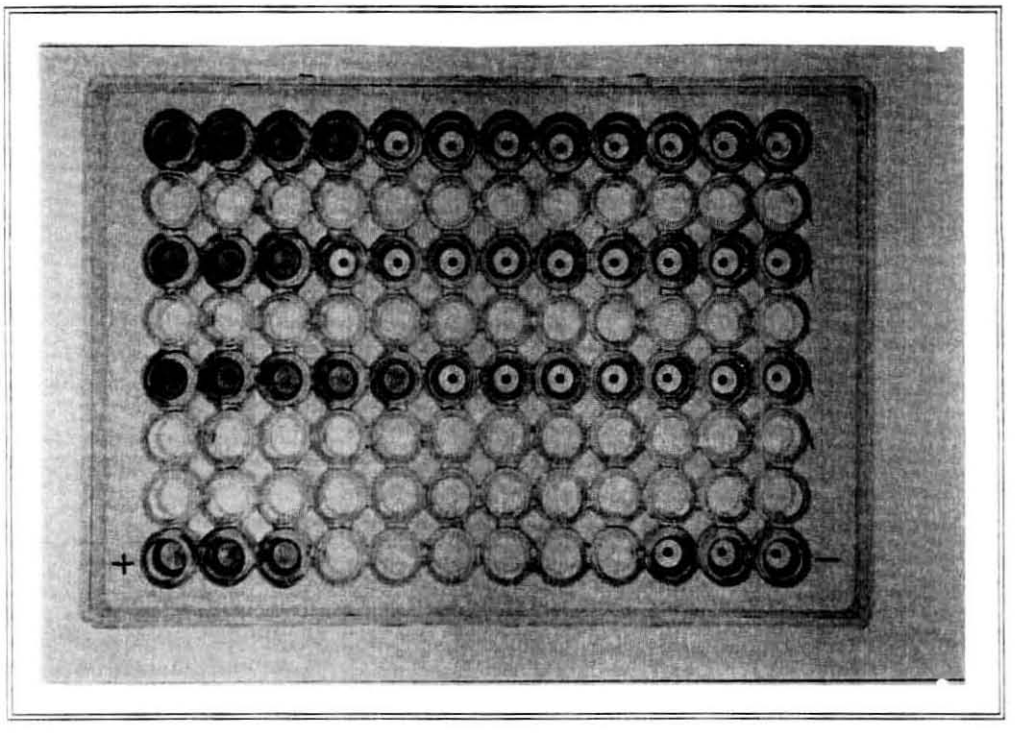


Plate 4

Showing hemolytic activity of partially purified skin toxins of *O. militaris* on chicken blood

Row 1: U4
Row 2: F6
Row 3: F10

+: Positive Control -:Negative Control

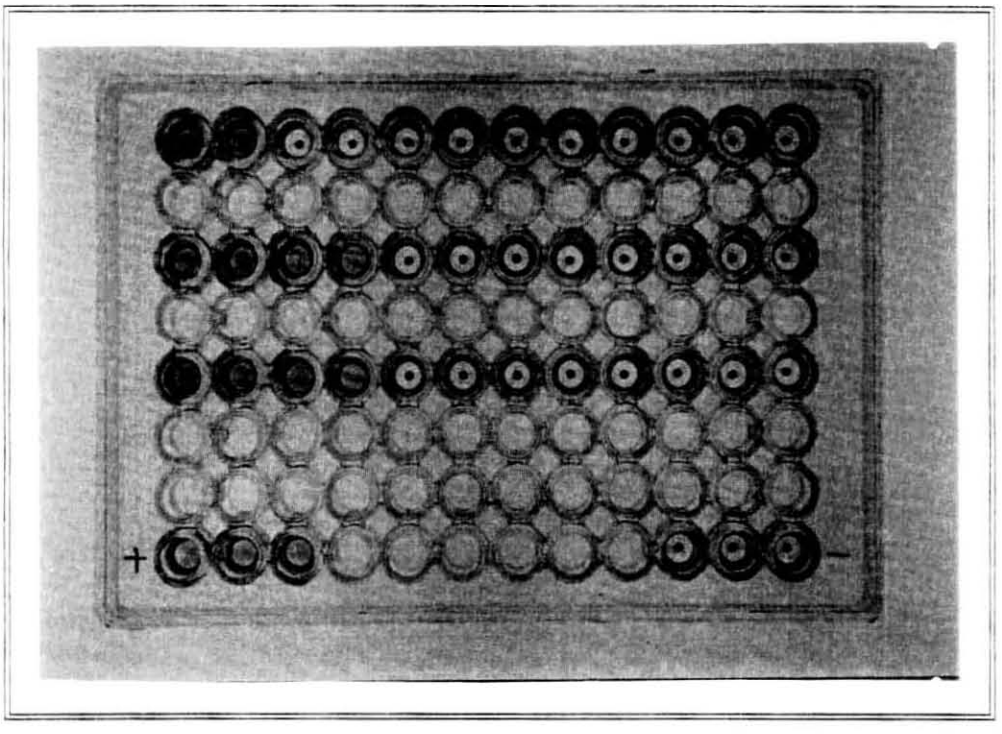


Plate 5

Showing hemagglutinating activity in partially purified skin toxin (Nov.97) of *A. dussumieri*

Row 1: F9
Row 2: F10

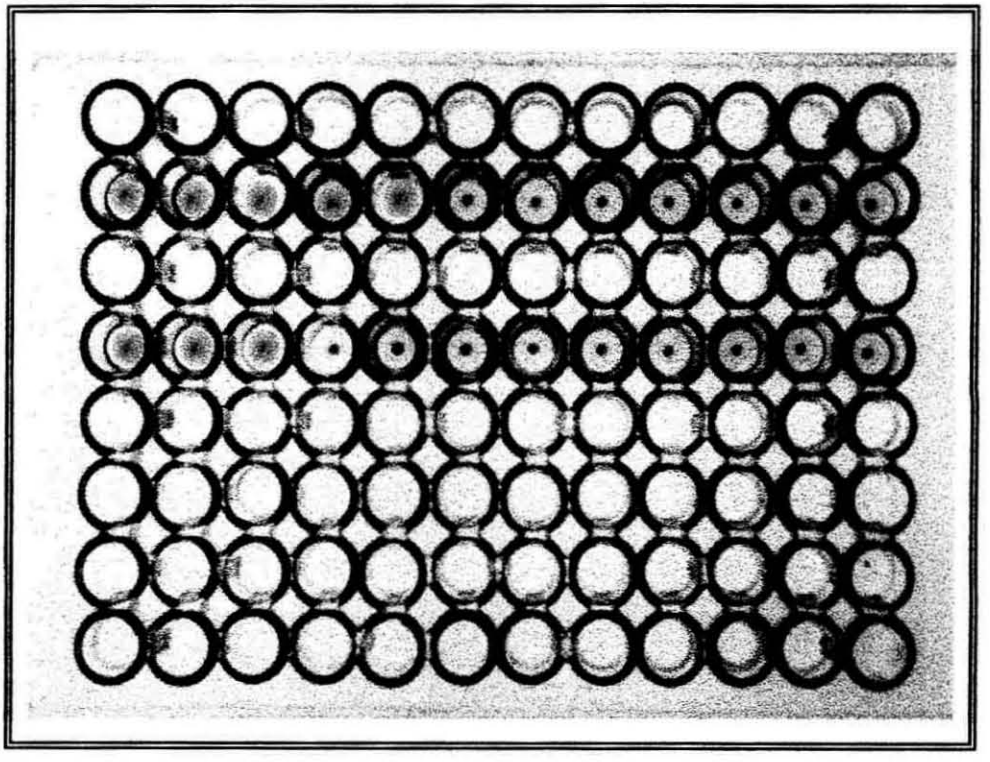


Plate 6

Showing hemagglutinating activity in partially purified skin toxin (Sep. 97) of *O. militaris*

Row 1: F8
Row 2: F9

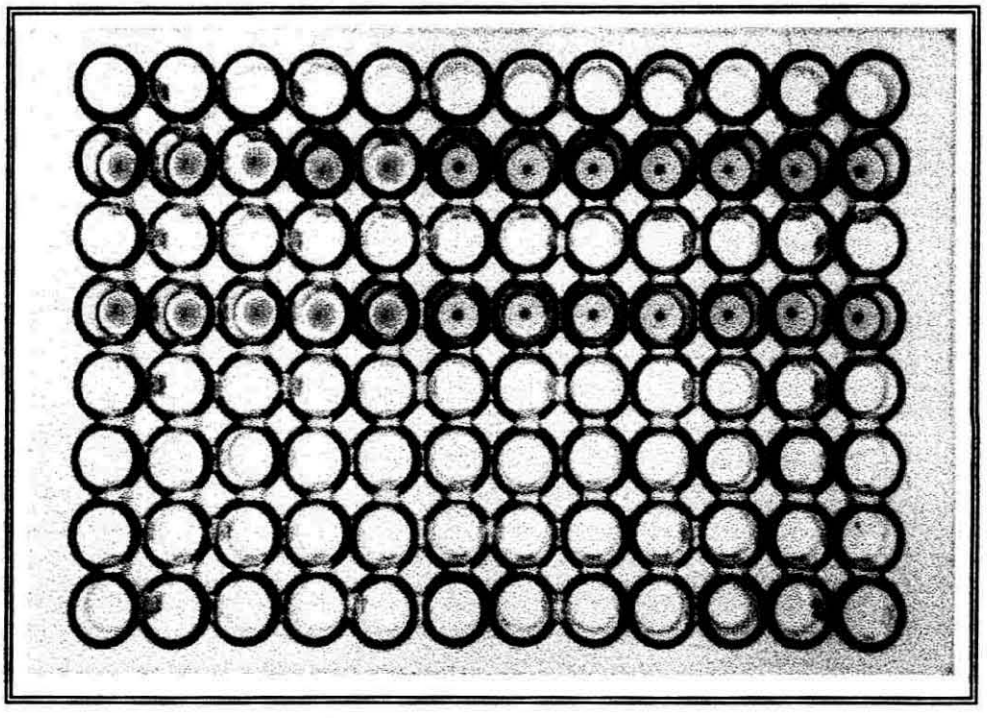
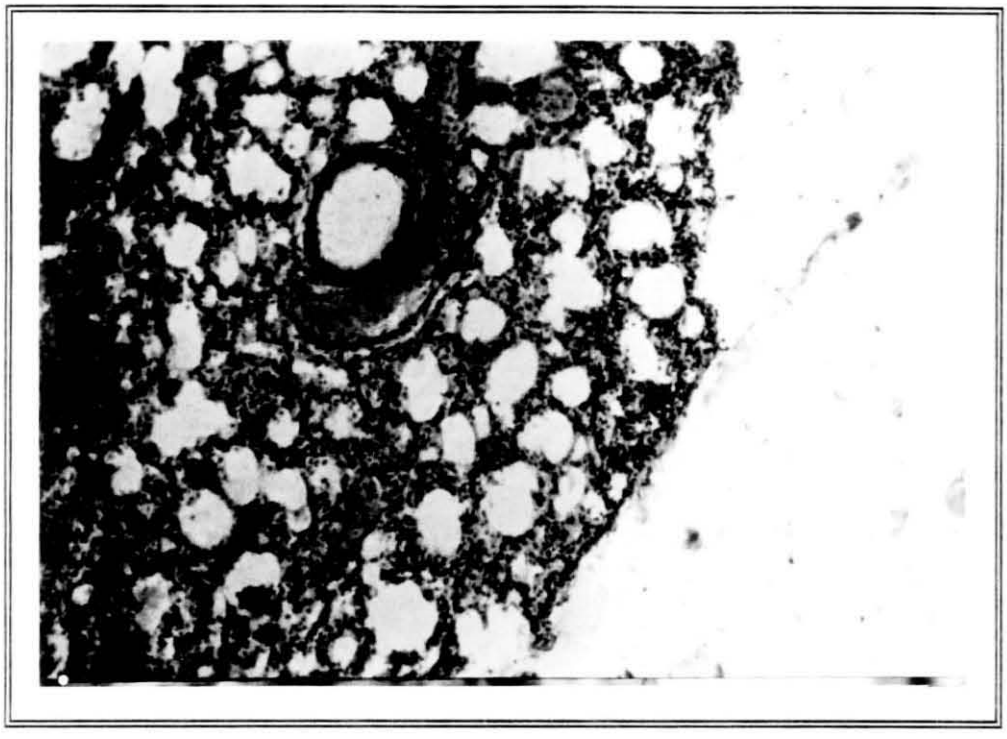


Plate 7

a. C.S. of lung of mice injected i.p. with 1 ml PBS (control) Hematoxylene-Eosin. x 100

b. C.S. of lung of mice injected i.p. with 1 ml of PBS (control) Hematoxylene-Eosin. x 400



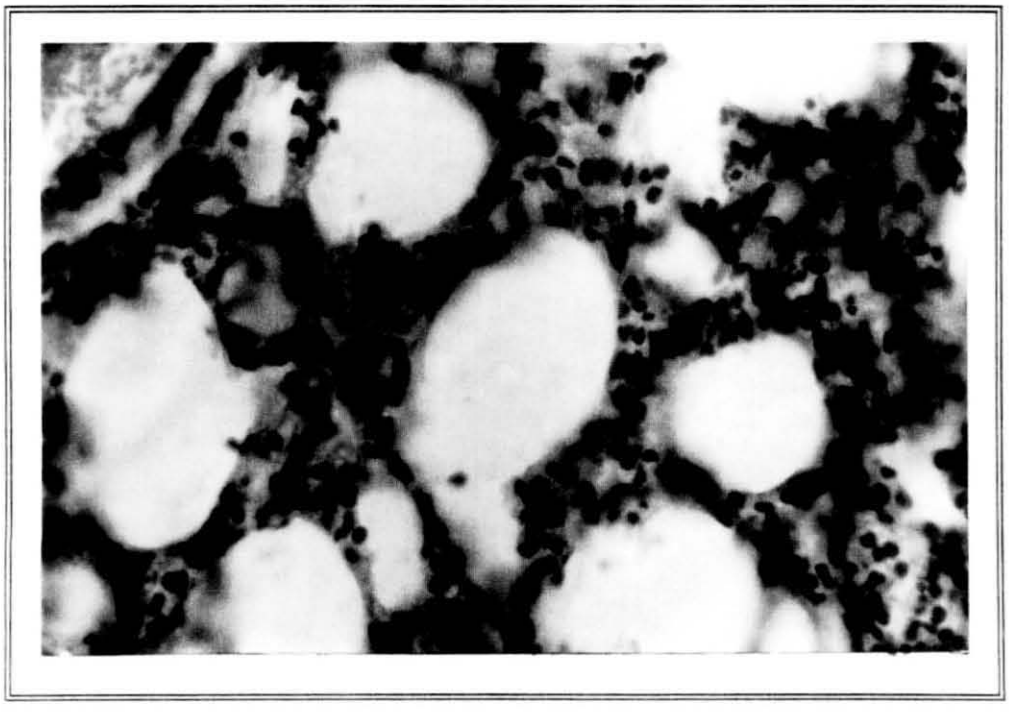
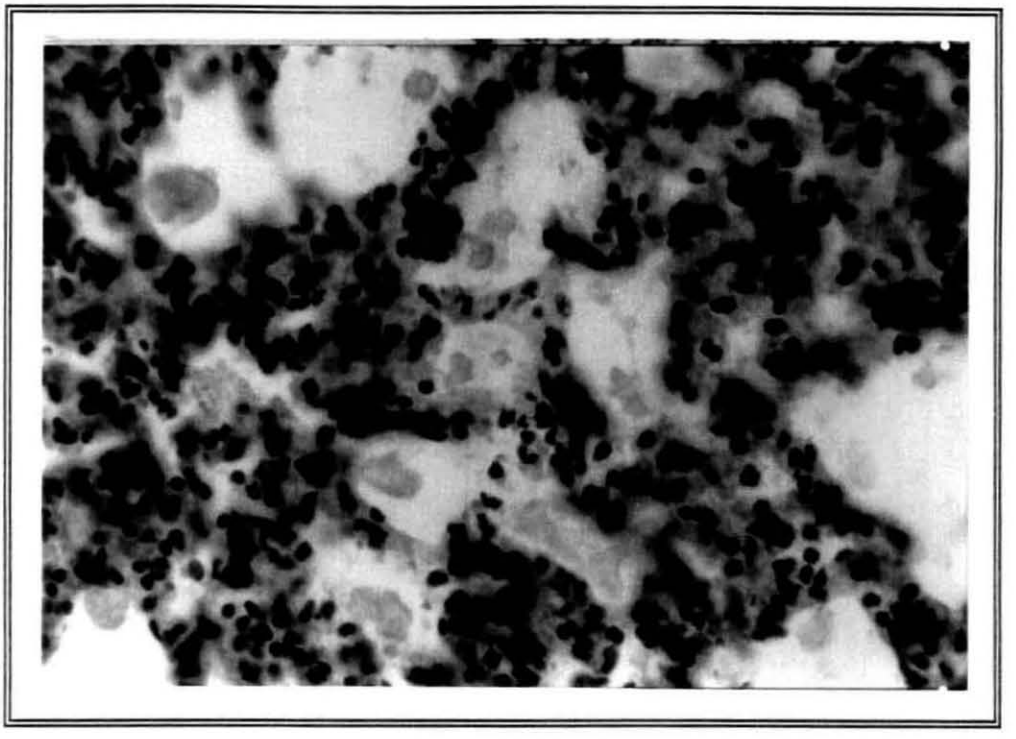


Plate 7

c. C.S. of lung of mice injected i.p. with 0.25 ml crude mucus toxin of *A. dussumieri.* Hematoxylene-Eosin. x 400

d. C.S. of lung of mice injected i.p. with 0.50 ml crude mucus toxin of *O. militaris.* Hematoxylene-Eosin. x 400



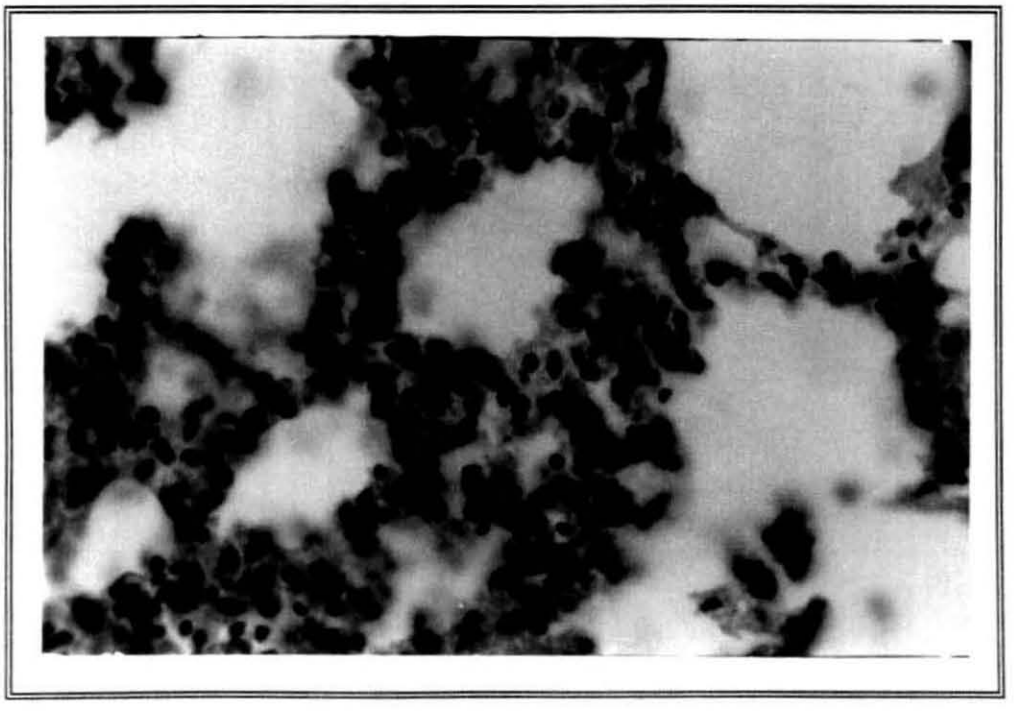


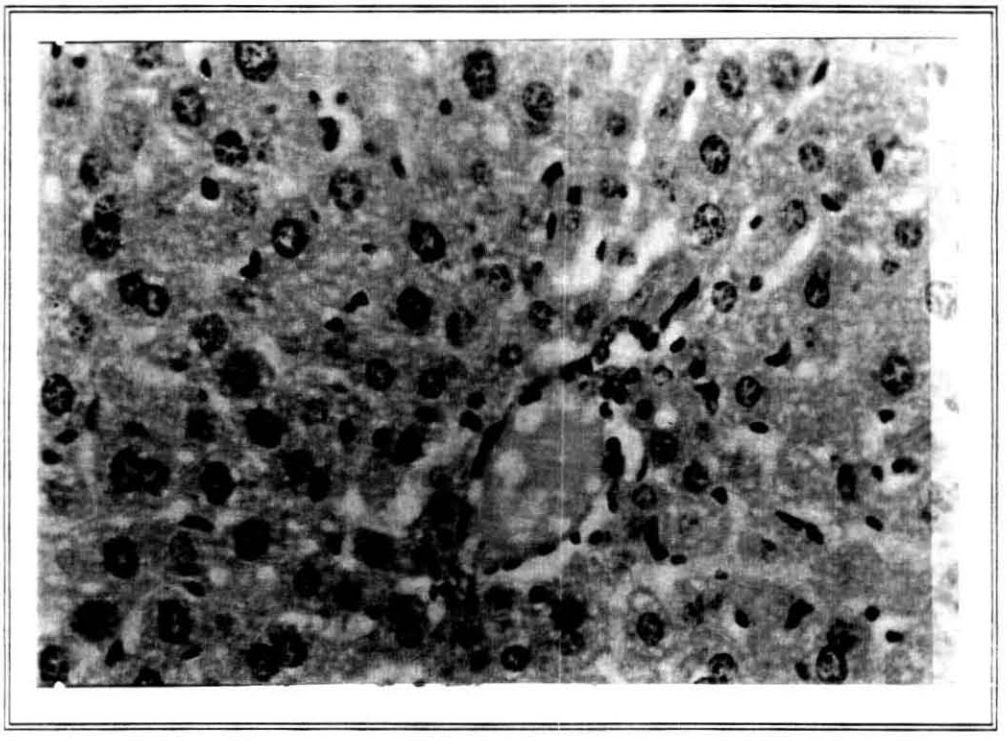
Plate 7

e. C.S. of lung of mice showing alveolar wall, injected with 1.0 ml of partially purified mucus toxin of *A. dussumieri*
Hematoxylene-Eosin x 1000

Plate 8

a. C.S. of liver of mice injected i.p. with 1.0 ml of PBS (contro) Hematoxylene-Eosin. x 400

b. C.S. of liver of mice injected i.p. with 1.0 ml of partially purified mucus toxin of *A. dussumieri.* Hematoxylene-Eosin. x 400



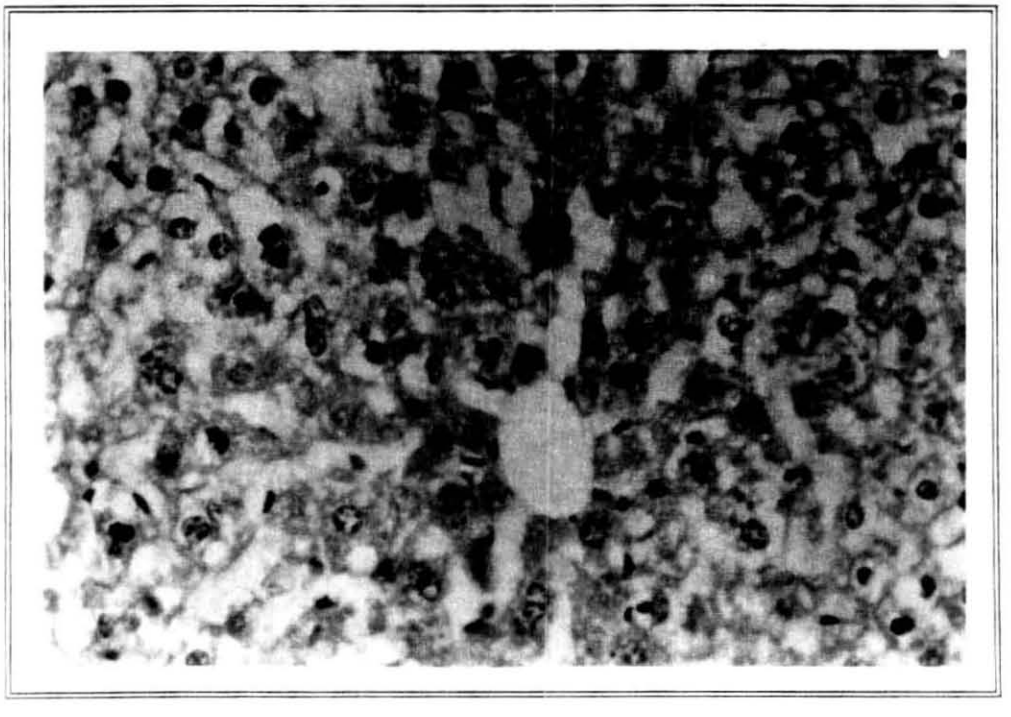
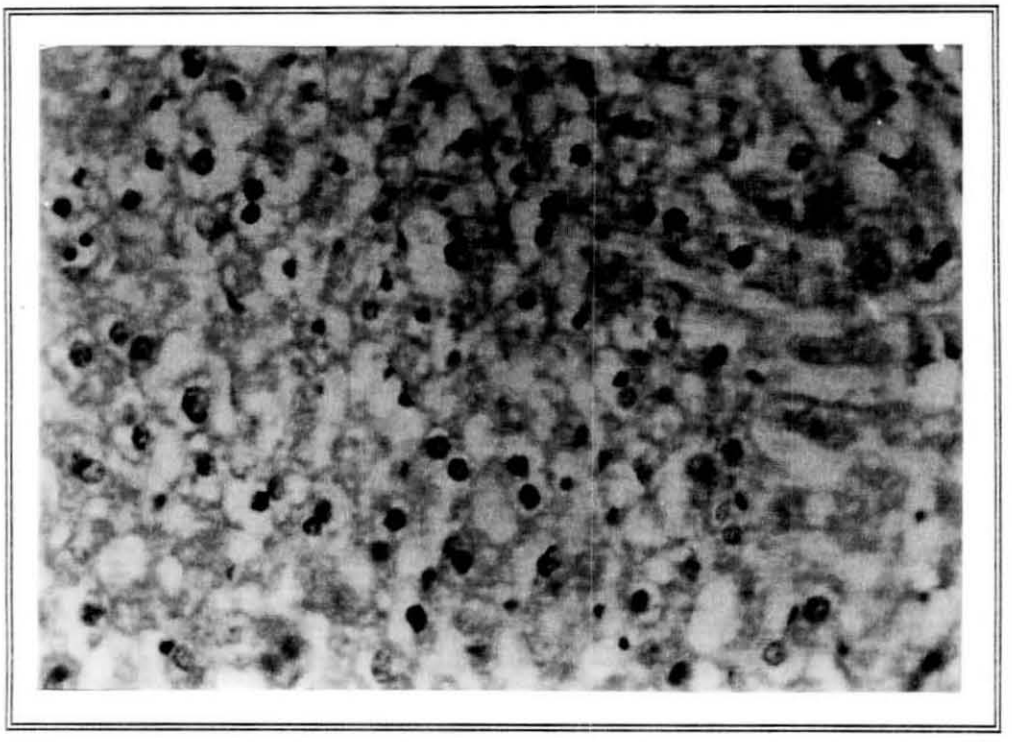


Plate 8

c. C.S. of liver of mice injected i.p. with 1.0 ml of partially purified mucus toxin of *O. militaris* Hematoxylene-Eosin. x 400

d. C.S. of liver of mice injected i.p. with 0.5 ml of crude mucus toxin of *O. militaris* Hematoxylene-Eosin. x 400



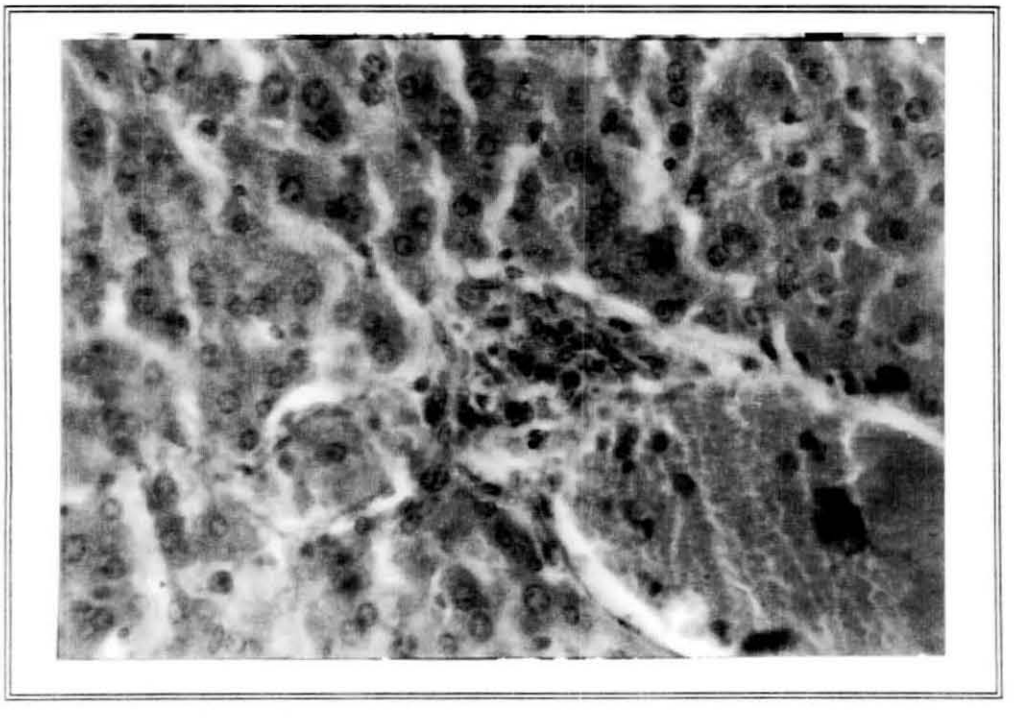


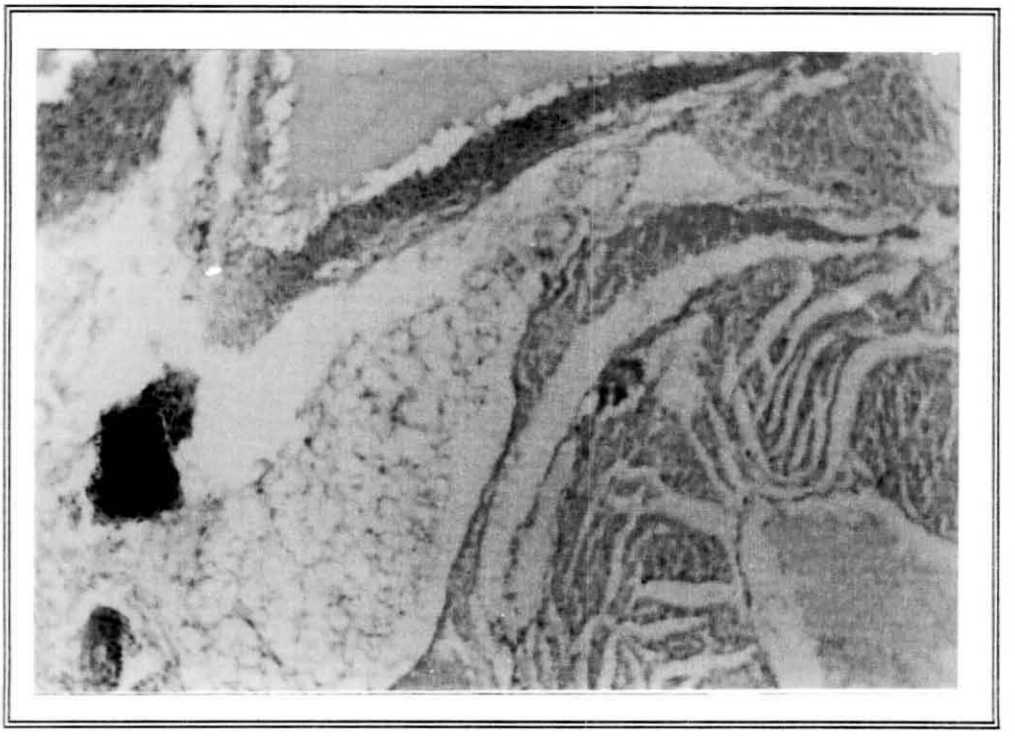
Plate 9

a. C.S. of heart of mice injected i.p. with 1.0 ml of PBS (control) Hematoxylene-Eosin. x 400

Plate 9

b. C.S. of heart of mice injected i.p. with 1.0 ml of partially purified mucus toxin of *A. dussumieri*. Hematoxylene-Eosin x 400

c. C.S. of heart of mice injected with 1.0 ml of partially purified mucus toxin of *O. militaris* Hematoxylene-Eosin x 400



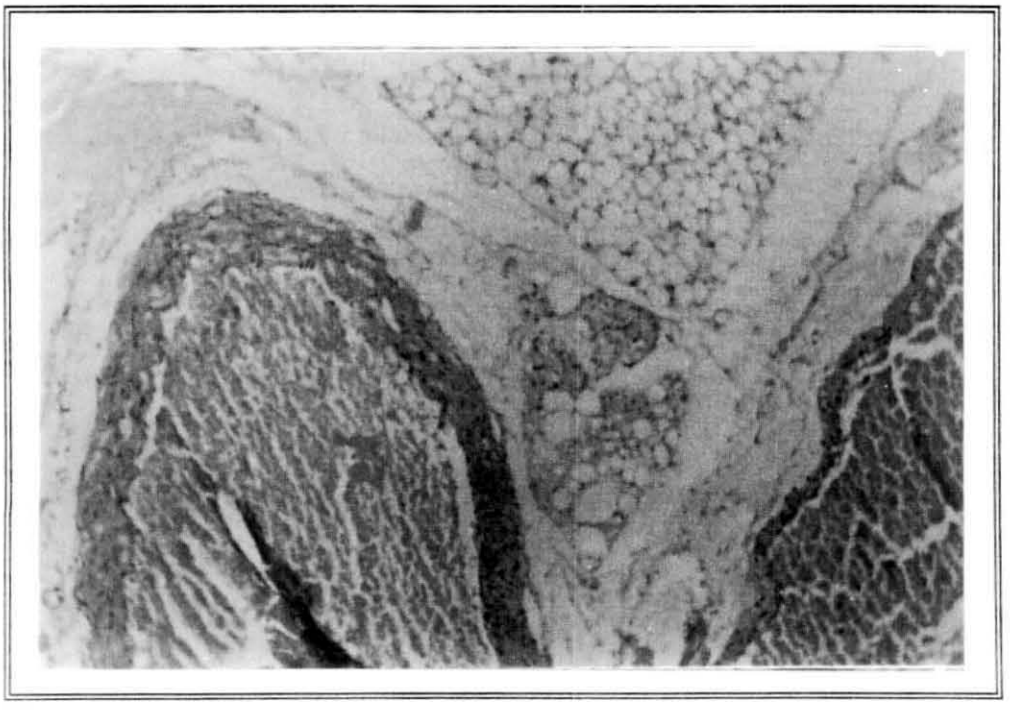


Plate 10

a. C.S. of kidney of mice injected i.p. with 1.0 ml of PBS (control). Hematoxylene-Eosin x 400

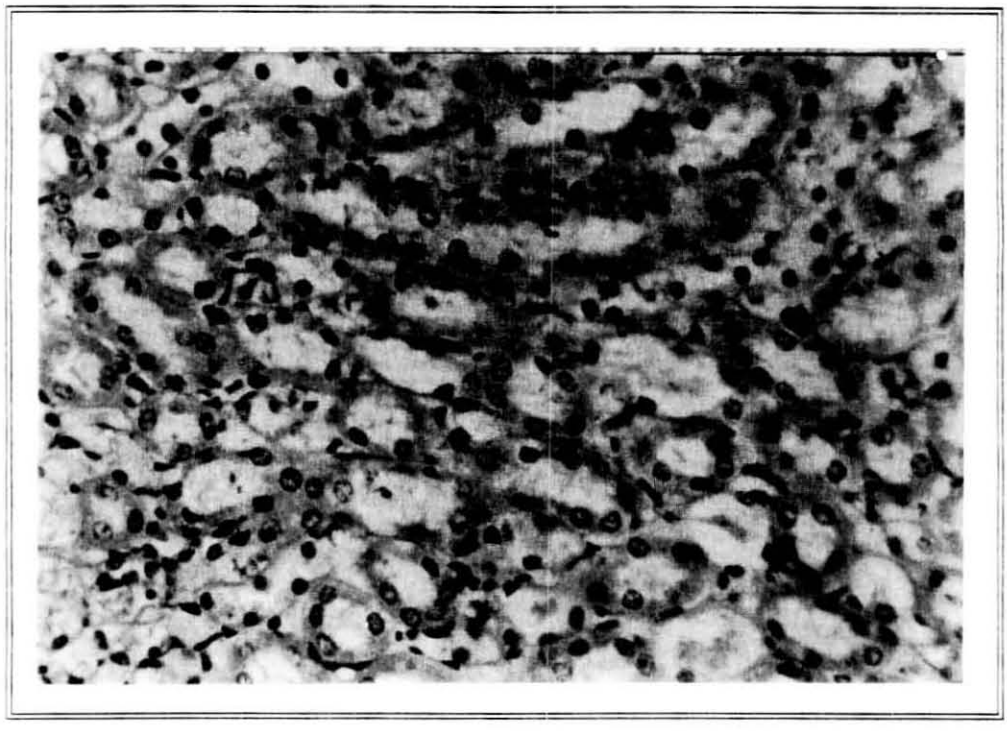
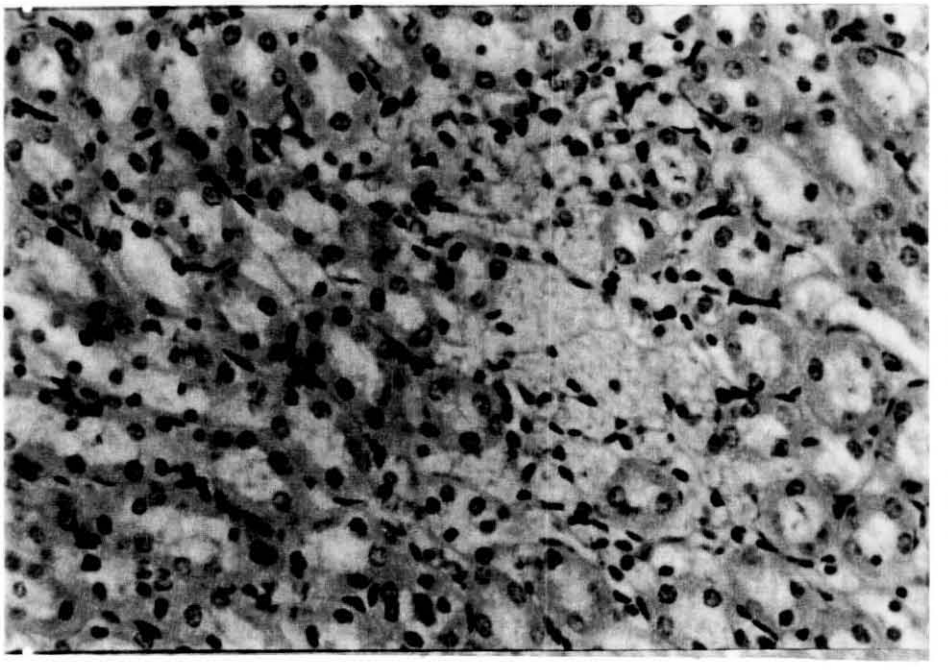


Plate 10

b. C.S. of kidney of mice injected with 1.0 ml of partially purified mucus toxin of *A. dussumieri*. Hematoxylene-Eosin. x 400

c. C.S. of kidney of mice injected with 1.0 ml of partially purified mucus toxin of *O. militaris*. Hematoxylene-Eosin. x 400



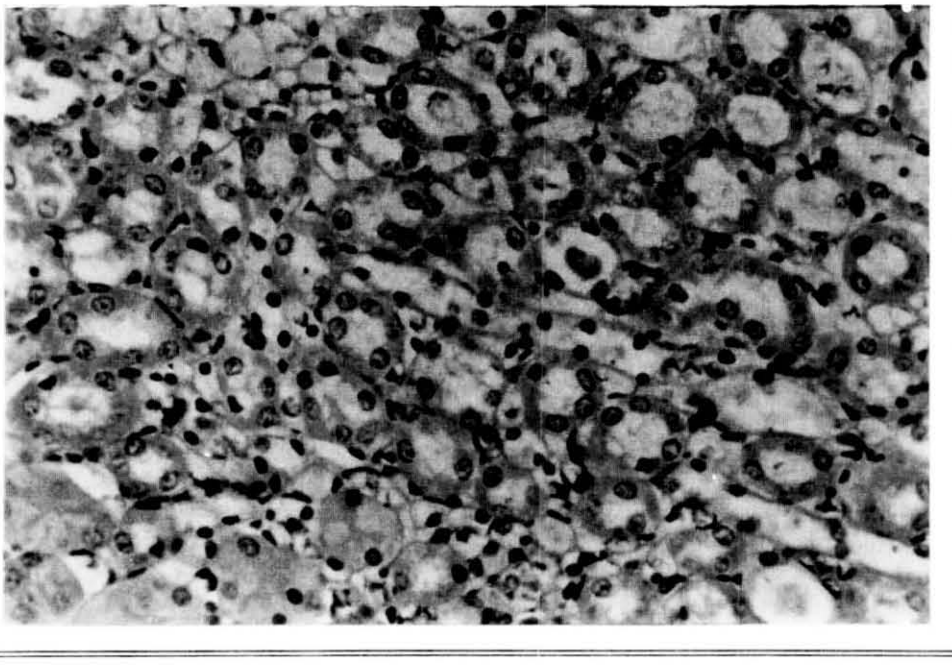
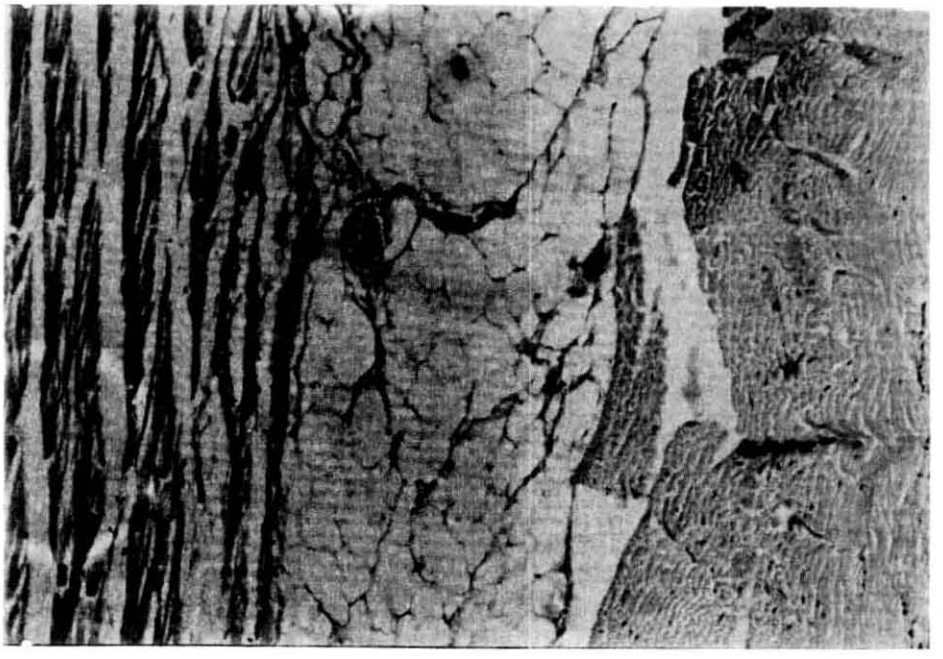


Plate 11

a. T.S. of skin of *A. dussumieri.* Hematoxylene-Eosin. x 400
b. T.S. of skin of *A. dussumieri.* Hematoxylene-Eosin. x 100



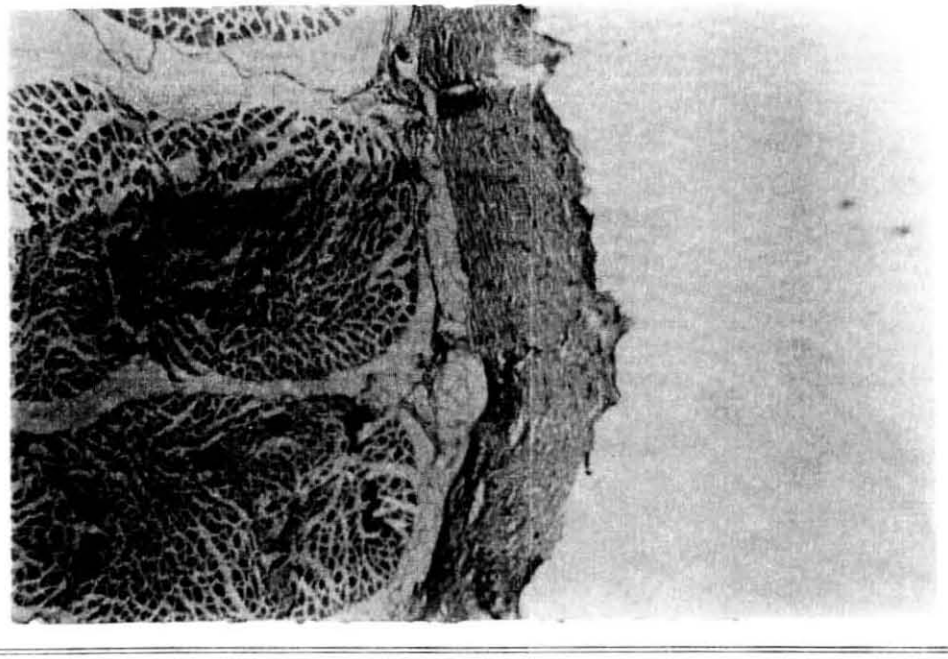


Plate 12

a. T.S. of skin of *O. militaris.* Hematoxylene-Eosin. x 400
b. T.S. of skin of *O. militaris.* Hematoxylene-Eosin. x 100

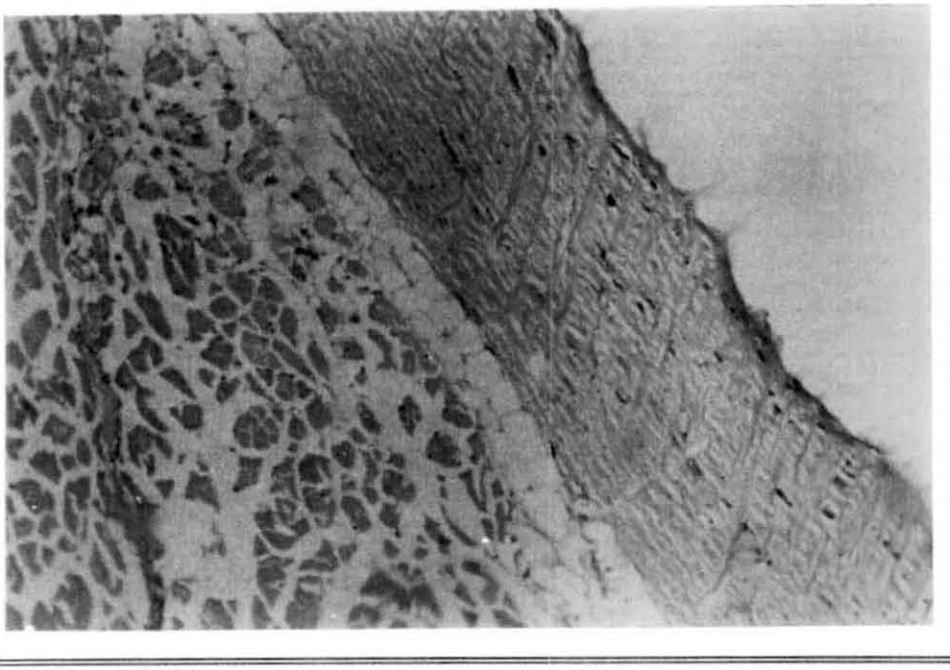
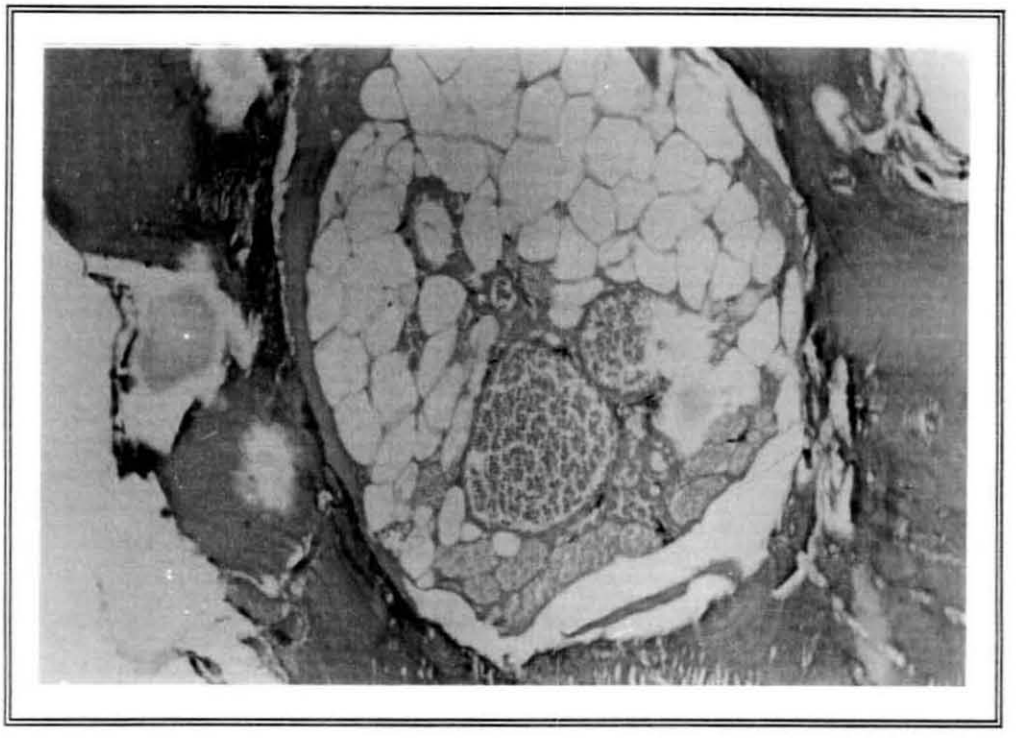


Plate 13

a. C.S. of pectoral spine of *A. dussumieri*. Hematoxylene-Eosin. x 400
b. L.S. of pectoral spine of *A. dussumieri*. Hematoxylene-Eosin. x 100



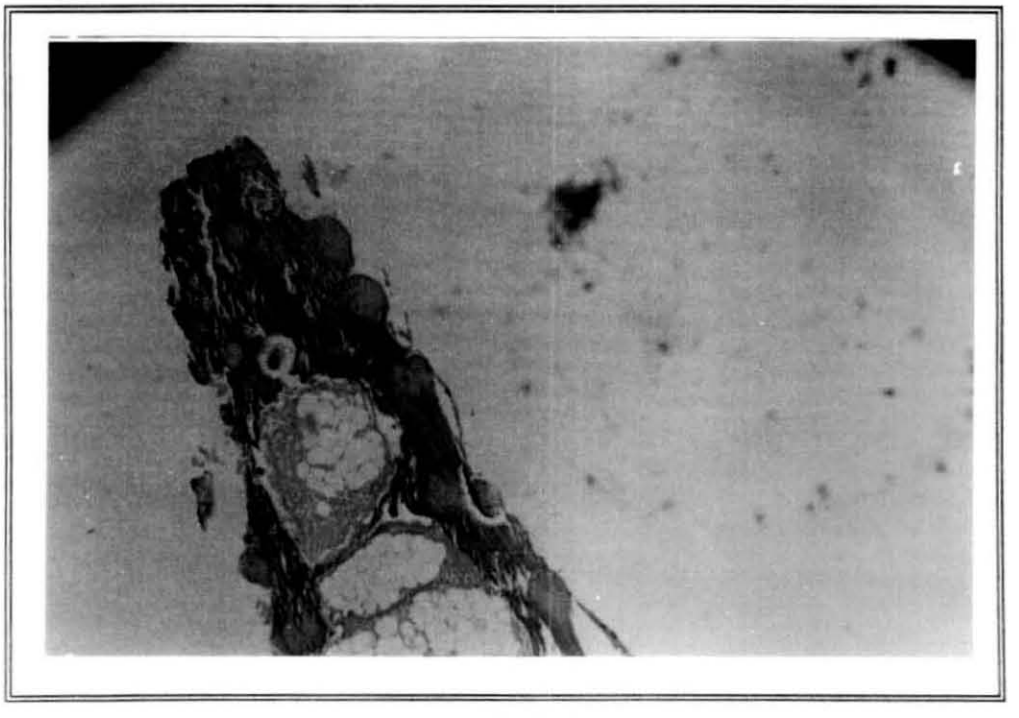
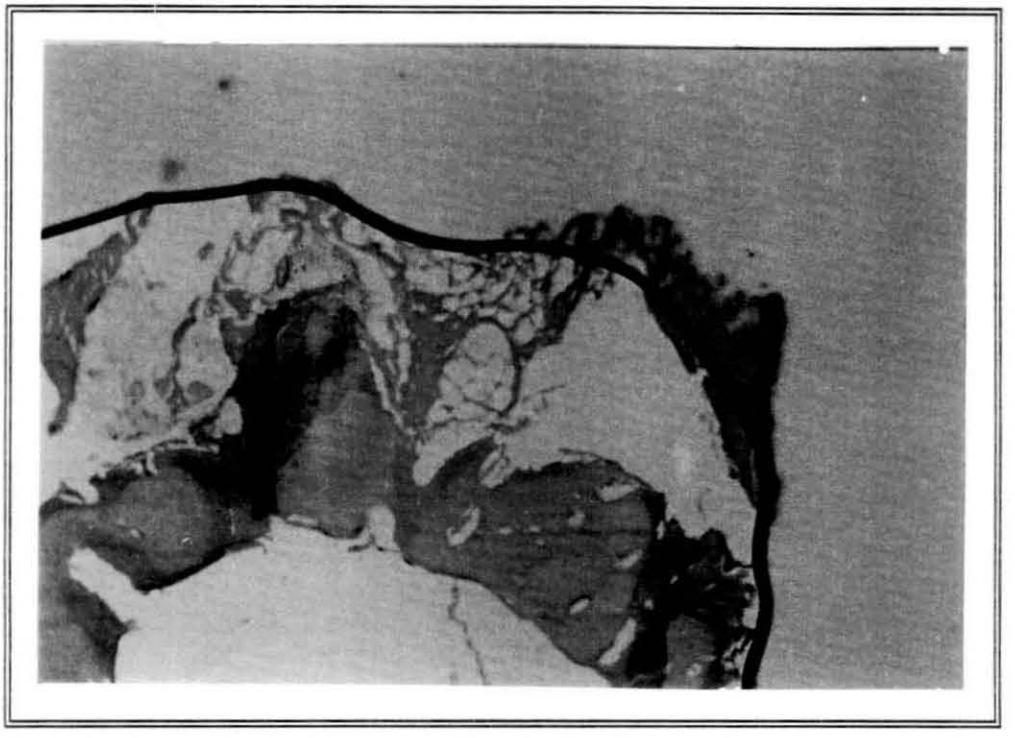


Plate 14

a. C.S. of pectoral spine of *O. militaris.* Hematoxylene-Eosin. x 100
b. L.S. of pectoral spine of *O. militaris.* Hematoxylene-Eosin. x 400



CHAPTER 4 : CHEMISTRY

4.1: Introduction

Marine biotoxions have received increasing attention from chemists and pharmacologists during the last two decades. Interest on the part of chemists has been two fold: natural products chemists have probed marine organisms as source of new and unusual organic molecules, while synthetic chemists have followed by targeting these novel structures for development of new analogs and new synthetic methodologies and strategies (Albizati *et al.* 1990). Marine toxins are complex and relatively dissimilar mixtures. The more toxic component of the fish venom is generally proteinous, although non-proteinaceous materials are also certainly present. Further the toxins from the other marine organisms are equally as unrelated in their chemical structure.

4.1.1: Chemical Structure

The pharmacological properties of the marine biotoxins appear to vary as remarkably as do their chemical properties. Some marine toxins provoke rather simple effects, such as transient vasoconstriction or vasodilation, while others provoke more complex responses. The effect of the separate and combined activities of the various components, and of the metabolites formed by their interactions, may be complicated by the response of intoxicated/ envenomated organisms. The organisms may produce and release several autopharmacological substances that can not only complicate the poisoning, particularly in man, but may also in themselves produce more serious consequences than the toxin (Albizati *et al.* 1990). The nature of the marine

toxins is further complicated by the fact that qualitative as well as quantitative differences in toxin may exist not only from species to species within the same genus but also from individual to individual within the same species. A toxin may even vary in its chemical and zootoxicological properties within the individual animal at different times of the year or under different environmental conditons (Russell and Brodie, 1974). The isolation and structure elucidation of new, structurally complex compounds have provided immense gratification to marine toxinologists and natural product chemists (Mc Conell *et al.* 1994).

The potential for utilization of bioactive/biotoxic agents of marine origin is great and a thorough understanding of their chemistry, structure-activity relationships and pharamacology of these agents will certainly lead to the synthesis of new drugs with highly selective actions and would prove an effective tool in pharmaceutical biomedical industry. Available information on chemistry and pharmacology of ichthyocrinotoxic fishes is very less except in case of trunkfish, boxfish, soapfish, sole, where chemistry of the skin extract, their activity and structure are well investigated and documented. However, such information on marine catfish, particularly from Indian waters, is scanty. In this back drop, the present chapter is aimed to find out the chemical nature of the toxin, its molecular weight determination and separation, characterization and also to know the fatty acid profile of two marine catfishes *Arius dussumieri* and *Osteogeneious militaris*, abundantly available along the Mumbai coast of India.

4.2: Review of Literature

Thomson (1964) isolated a crude toxin from the skin of boxfish, *Ostracion lentiginosus*, which was nonproteinous and nondialyzable. Boylan and Scheuer (1967) extracted a toxin from the same fish with butanol and purified it by silica gel column chromatography, and finally collected the active fraction in a white powdery form that was identified as the choline chloride ester of 3-acetoxyhexadecanoic acid. They found that this crystalline form of pahutoxin melts at 75^0C and is insoluble in water, ethanol, chloroform, hot acetone and hot ethyl acetate. The infrared spectrum of pahutoxin in chloroform solution showed the presence of quarternary nitorgen, saturated hydrocarbon and ester function. The NMR spectrum in deuteriochloroform confirmed the presence of a large aliphatic portion. By hydrolytic degradation and synthesis, its molecular formula was found to be $C_{23}H_{46}NO_4$-Cl.

Goldberg *et al.* (1982) isolated an ichthyocrinotoxin from the skin secretion of the smooth trunkfish, *Lactophrys triqueter* in crystalline form and identified it as the choline chloride ester of palmitic acid. The major component of this toxin was identified to be the choline ester chloride of heptadecanoic acid.

Goldberg *et al.* (1984) identified homologous fatty acid choline esters from trunkfish *L. triqueter* by methane chemical ionization gas chromatography mass spectrometry and found $(M\text{-}CH_3Cl)^+$ as their highest mass ion and $CH_2\text{-}N^+(CH_3)_2$) as the base peak. They also subjected B-actoxy palmitylcholine chloride (pahutoxin) for mild hydrolysis that yielded B-acetoxy palmitic acid.

Working with Japanese boxfish *Ostracion immaculatus*, Fusetani and Hashimoto (1987), obtained mucus secretion in butanol and fractionated it by

silica gel column chromatography and reversed phase high performance liquid chromatography (HPLC) in to two hemolytic components; the first major hemolysin was identical with pahutoxin and the second was a new choline chloride ester, named homopahutoxin.

Goldberg *et al.* (1988) studied the nature of the toxic skin secretion from eight species of trunkfish (Family- Ostraciidae) by NMR and found that the predominant component is substituted choline chloride esters of palmitic acid in all cases.

Maseretski and Castillo (1967) isolated a toxin from the soapfish *Rypticus saponaceous* and, from gel filtration and dialysis experiments, concluded that it was a high molecular weight polypeptide. Crude toxin solutions at neutral pH were observed to undergo rapid inactivation but remain stable at pH 3-4. Hashimoto and Oshima (1972) separted three preparations, grammistins A, B and C from the butanolic extract of soap fish *Pogonoperca punctata* by counter current distribution and subsequent column chromatography on Sephadex LH-20. Final purification was effected by carboxymethylcellulose chromatography that yielded two components, A_1 and A_2 from grammistin A. Grammstin A_1 and A_2 differed from each other, but both were about 80% peptidic, consisting of 24 amino acids. Grammistins A, B and C from other three species of soapfishes, *Grammistes sexlineatus, Diploprion bifasciatum* and *Aulacocephalus temmincki* were compared with grammistins of *P. punctata*, the latter showed a close relationship with those from *G. sexlineatus.* Grammistins of *D. bifasciatum* and *A. temmincki* revealed a different pattern of elution and distribution from those of *P. punctata.* The major grammistin from *A. temmincki* was observed to be a very large

molecule. The hemolysin of *D. bifasciatum* was fat soluble, without a peptide moiety (Oshima *et al.* 1974).

Hori *et al.* (1979) found the skin secretion of clingfish, *Diademichthys lineatus* to be very much similar to grammistin based on by gel filtration studies and dialysis. The amino acid analysis of the toxin indicated the presence of a large non-peptide moiety that contained a Dragendorff- positive group. They proposed the name gobiesocin for this toxin.

Shiomi *et al.* (2000) separated two fractions, GS_1 and GS_2 from aqueous skin extract of soapfish *G. sexlineatus* by gel filtration on Sephadex LH-20 and subsequently by reverse phase HPLC on an ODS-12OT column. The complete amino acid sequences of GS_1 comprising 25 residues and GS_2 comprising 24 residues were determined. Further investigation revealed that grammistin GS_2 was much more abundant in amphiphilic α- helices and much higher biological activity than grammistin GS_1.

Nair *et al.* (1982) isolated an active fraction from butanolic skin extract of toadfish *Opsanus tau* by TLC and low-pressure chromatography on flash silica gel column. The toxin fraction was analyzed by gas chromatography-mass spectrometry (GC-MS) and was found to be a mixture of three aliphatic amines. The main toxic component was characterized as 3-octanone.

Primor and Zoltkin (1975) separated two active fractions by Sephadex G-75 from the toxic skin secretion of *Pardachirus marmoratus* and found both the fractions to overlap each other qualitatively as well as quantitatively suggesting that both the fractions were involved in toxic action.

Primor *et al.* (1978) separated a peptidic ichthyotoxin, pardaxin (PX) from *P. marmoratus*, which was a mixture of two closely related peptides,

pardaxins PX-I and PX-II. Thompson *et al.* (1986) isolated from *P. pavoninus* three peptides having similar ichthyotoxic, hemolytic and shark-repellent properties and named them pardaxins P-1 to P-3. Further, they separated these three pardaxins by gel filtration and ion-exchange chromatography, and final purification was effected by reversed phase HPLC. Upon amino acid sequencing these peptides were found to consist of 33 amino acid residues with hydrophilic carboxyl terminal and hydrophobic remainder. They also found similarities in the physical and biological properties of these peptides with the bee venom, melittin.

Lazarovici *et al.* (1986) reported the isolation of two pardaxins, PX_1 and PX_2, in pure form from the Red Sea Moses sole *P. marmoratus*, using a combination of ion-exchange chromatography, chromatofocusing and reverse phase HPLC. They observed a high degree of homogeneity and resemblance between the two toxins, by amino acid analysis, and NH_2 -terminal amino acid sequence. The toxins were rich in aspartic acid, serine, glycine and alanine but devoid of arginine, tyrosine and tryptophan. Nakagawa *et al.* (1988) separated glycopeptides from 13 species of fishes and analyzed them for their carbohydrate components.

Histochemical investigations revealed the external mucus of eel, *Anguilla joponica* to be acidic; the carbohydrate moiety of the SA-glycoprotein was attached to the protein by an alkali-labile linkage, as O-glycosides of threonine and serine residues and was a dissacharide composed of H-acetylneuraminic acid (NANA) and N-acetylgalactosaminitol (Asakawa, 1974, 1977, 1979).

Enomoto *et al.* (1966) reported the presence of sialic acid in the external mucus of three species of cartilaginous fishes and one species of eel by paper chromatography. Mucus-polysaccharide of seven kinds of fishes was qualitatively analysed and found to have the same component sugar irrespective of the difference in their living medium and in classification system (Enomoto and Tomiyasu, 1961, 1962). Further they found out the neutral sugar composition qualitatively by column chromatography using Dowex-1 and concluded that hexose was predominant. The components of basic sugars were found to be galactose amine and glucosamine and that of acidic sugar was glucuronic acid (Enomoto *et al.* 1963).

Lyophilized toxic secretions of *P. pavoninus* were dissolved in 0.1M aqueous ammonia and precipitated by adding acetone. The supernatant was partitioned between ehtyl acetate and water. Removal of the ethyl acetate yielded a type of oil, which was chromatographed repeatedly to yield pure form of pavonins 1-6 (Tachibana *et al.* 1984). Structure of pavonins was determined as steroid N-acetylglucose aminides, by spectroscopic studies and correlation to known compounds (Tachibana *et al.* 1983). They suggested that the pavonins were responsible for the repellent property against any predatory fishes. Kimura *et al.* (1994) characterized a sialic acid containing glycoprotein as 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN) using NMR spectroscopy and mass spectrometry from the mucus of the loach, *Misgurnus anguillicaudatus*. A sialoglycoprotein was isolated and further characterized by using TLC, GLC, and gel filtration from the skin mucus of stingray, *Dasyatis akajej* (Sumi *et al.* 1997 *a, b*).

Toda *et al.* (1996) reported a mitogenic lectin in the mucus of the king lip *Genypterus capensis*. The lection was a glycoprotein having a molecular weight of 28-34 kDa that was composed with two homogenous subunits of 13.7 kDa. The hemagglutinating activity was found to be calcium ion dependent and only seen in rabbit and horse erythrocytes but not against human erythrocytes. The active components of mucus of moray eel *Lycodontis nudivomer* showing hemolytic, toxic and hemagglutinating activities were separated with 0.02 M phosphate buffer (pH 7.5) and purified by DEAE-cellulose and Sephadex G-200 column chromatography. The toxic-hemolysin component was unstable in the presence of heat, acidic and alkaline and several organic solvents. It was undialyzable through a cellophane membrane, and found to be a protein, with a molecular weight more than 100,000 (Randall *et al.* 1981). Suzuki (1985) analyzed the mucus extract of Japanese eel, *Anguilla japonica* by gel filtration on Sephacryl S-400 column and calculated its molecular weight as 370,000 and 290,000 for hemogglutinin and hemolysin respectively.

Shiomi *et al.* (1990) extracted the mucus toxin of *A. japonica* in phosphate buffer saline and chromatographed on HPLC using TSK-gel G 3000 SW column, calibrated with ferritin, aldolase, bovine serum albumin, ovalbumin and ribonuclease A as reference proteins; they found a molecular weight of 40,000-50,000 for hemagglutinin and more than 200,000 for the toxin. The soluble skin extract of *Plotosus canius* when fractioned by gel filtration chromatography on Sephadex G-50 followed by ion-exchange on DEAE-Sephacel, which yielded two major proteins, hemolysin (Plolysin) and a toxic

protein (Plotoxin) with identical molecular weight of 3,300 on SDS-PAGE (Othman *et al.* 1993).

Shiomi *et al.* (1987) purified one lethal factor from the skin secretion of oriental catfish *Plotosus lineatus* successively by DEAE-cellulose and Sephadex G-75 chromatography. The molecular weight was found to be 10,000 by gel filtration on sephadex G-75. They pointed out that the purified lethal factor was a basic protein, with more numbers of acidic amino acids than basic amino acids, indicating that the acidic amino acids exist in the molecule in an amide form. Earlier, Shiomi *et al.* (1986) reported one hemolysin and two identical lethal-edema forming factors in the mucus secretion from the same species; molecular weight of the hemolysin and the lethal factors (edema-forming factors) were estimated to be 180,000 and 12,000 respectively. Al-Hassan *et al.* (1982) have indicated that the major portion of the gel secretion from the skin of the Arabian Gulf catfish, *Arius thalassinus* was protein (85%) followed by lipid (13%) with very little carbohydrate (1.4%) and the rest nucleic acid, etc. The soluble epidermal gel of *A. thalassinus* contained at least two components that could stimulate muscle contraction. The effect of one component was blocked by atropine pretreatment, indicating that it was acetylcholine-like in action. The substance was heat stable, non-proteinous, and had low molecular weight (Al-Hassan *et al.* 1986*a*). In a subsequent study Al-Hassan *et al.* (1986*b*) separated a galactose-specific lectin by exclusion chromatography, affinity chromatography and isoelectric focussing. The lectin had a single polypeptide chain with a molecular weight of nearly 200,000 that could form oligomers and hetropolymers. It formed 2% of the total gel protein and had no carbohydrates with unusual or amount of amino acids. Al-Lahham

et al. (1987) purified a protein having a molecular weight of 34,000 by molecular sieve chromatography and SDS-PAGE. This protein was found to have a very strong hemolytic action against various erythrocytes tested. Al-Hassan *et al.* (1987) studied the protein composition of the threat induced epidermal secretion from the Arabian Gulf catfish, *A. thalassinus* and found not much difference in the amino acid profile of soluble and insoluble fractions. Both the soluble and insoluble fractions had aggregating proteins.

The shock-induced epidermal secretions from the fish *Arius bilineatus* contained two classes of esterase activities. The first class of esterase activity catalyzed hydrolysis of the general esterase substrate, P-nitrophenyl acetate and second is specific of esters for tyrosine. Purified tyrosine specific esterase was a serine type esterase with a molecular mass between 70,000 and 100,000 Daltons, but had no amidase activity (Thomson *et al.* 1989) Thomson *et al.* (1998) isolated and purified a toxic factor from the crude skin extract of *A. bilineatus* by a combination of gel filtration on Sephacryl S-300 and preparative discontinuous PAGE, with a molecular weight of 39,000 Da and isolectric point (pI) of 5.45.

Ali *et al.* (1989) studied the comparative biochemical and pharmacological properties of epidermal secretions from three ariid catfishes, *A. bilineatus, A. tenuispinis* and *A. thalassinus* and concluded that the protein composition was generally similar, except for the size and number, whereas lipid compositions varied significantly with the season winter and summer, but the enzymes appeared to be common in each fish with slight variation in their respective activities.

4.3: Material and Methods

Chemical analysis of the mucus extracts that had shown the highest level of toxicity was done under three different processes. In the first and second processes, proteinous component was analysed by SDS-PAGE and HPLC, whereas in the third step lipid component of the mucus was analyzed by gas-chromatography.

4.3.1: Separation of Proteins from the Crude Mucus Extract

Protein was separated by precipitating crude mucus extract in Trichloro acetic acid (TCA). An equal volume of mucus extract and 10% TCA was mixed and kept at 4^0 C for overnight and then centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and the pellet left at the bottom was used as protein component.

i) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Electrophoresis of protein from the crude mucus were carried out by the method of Laemmli (1970) in 12% polyacrylamide slab gels, using reagents as described in Annexure 4. 60 µg of the separated protein in each case was diluted with sample buffer (1:4) and heated at 95^0 C for 3 minutes, then loaded.

The wide range molecular weight markers (Sigma) used were Myosin, Rabbit Muscle (205 KDa), ß-Galactosidase, *E. coli* (116 KDa), Phosphorylase-b, Rabbit muscle (97 KDa), Fructose-6-phosphate, Kinase, Rabbit muscle (84 KDa), Albumin, Bovine Serum (66 KDa), Glutamic Dehydrogenase, Bovine liver (55 KDa), Ovalbumin, Chicken Egg (45 KDa), Glyceraldehyde-3-phosphate Dehydrogenase, Rabbit muscle (36 KDa), Carbonic Anhydrase, Bovine

erythrocytes (29 KDa), Trypsinogen, Bovine pancreas (24 Kda), α-lactalbumin, Bovine milk (1.4 Kda), Aprotinin, and Bovine lung (6.5 Kda). The volume of marker loaded was 3 µl and 5 µl respectively in the extreme left and extreme right wells. The wells in between these two markers were loaded with the samples of proteins. Electrophoresis was run at 25 mA per slab gel. Upon completion of electrophoresis, gel was washed gently with tap water to remove excess SDS, stained in Comassie Blue R250 (Coomassie brilliant blue R250, 1.25 g; Methanol 227 ml; Glacial acetic acid 46 ml; Distilled water to make up to 500 ml) for two hour at room temperature and then destained (Methanol 7 ml; Glacial acetic acid 7 ml; water to make up to 100 ml) for 48 hours. Protein bands were visualized as dark blue bands on a light blue background.

ii) Analysis of lethal factor by High Performance Liquid Chromatography (HPLC): HPLC analysis of the lethal factor of both the species were done on a Hewlett Packard HPLC system (1050 series) following the method of Lazarovici *et al.* (1986) with slight modification. Acetonitrile and Water formed the mobile phase with Trifluroacetic acid (TFA) as the solvent modifier. The protein standard used was from Sigma, USA. A Hewlett Packard Hypersil C_{18} (200 x 4.6 mm) BDS Column of 5 µm particle size and 130 A^0 pore diameter, and a UV detector (Hewlett Packard 1050 Series) were employed. The run conditions were as follows:

Time (min)	**% Acetonitrile containing 0.1% TFA**	**% Water**	**Flow rate (ml/min)**	**Abs (nm)**
0	15	85	0.9	226
20	25	75	0.9	226
50	85	15	0.9	226

Injection volume: 20 µl and Run time: 50 min.

iii) Analysis of Lipid Component: Analysis of lipid component was done by Gas chromatography. Lyophilized mucus extracts of both *A. dissumieri* and *O. militaris* were mixed with the mixture of chloroform methanol (2:1 -V/V) using a vortex mixer and was then centrifuged at 5,000 rpm for 3 minutes. The supernatant was transferred into other vial and evaporated till dried. Residue thus obtained was treated as lipid sample.

Esterification of lipid: In the first step for Gas chromatography, a methyl ester (FAME) of the sample was prepared; the lipid sample, sodium hydroxide, methyl alcohol, boron tri fluoride (BF_3) and heptane were mixed together following Method 969.33 of AOAC (1990). An aliquot of the upper heptane solution was removed and dried with anhydrous Na_2SO_4. The dried matter was diluted to a concentration of 7% for injection on Gas chromatograph (Chemito 8610 fitted with DEGS column).

Temperature was kept 60^0 C for 4 minutes, then raised to 200^0 C at a rate of 5^0 C/min and held at 200^0 C for 5 minutes. Injector and Flame Ionization Detector (FID) temperatures were 280^0 C and 300^0 C respectively. Oven temperature was maintained at 220^0 C.

The carrier gas (N_2) flow rate was 23.1 ml/min while Hydrogen flow rate to the detector and airflow rate to the detector were maintained at 23.2 ml/min and 300 ml/min respectively. Sample size was 0.5 µl and the total run period was 18 minutes. Identification of fatty acids was carried out on the basis of retention times of the standard mixture of fatty acids.

4.4: Results

4.4.1: SDS-PAGE

Upon SDS-PAGE on 12% gel, crude mucus extract of *A. dussumieri* and *O. militaris* yielded 18 and 16 bands respectively, ranging from 10 kD to 100 kD with prominent bands at 14 kD and 27 kD in both cases (Plate 11).

4.4.2: HPLC Analysis

The combined lethal factor in both species showed a prominent peak of elution at 25 minutes of Retention Time, resembling closely that of Cytochrome C of the Protein Standard (Sigma). This fraction also elicited the lethal effects when tested on mice. The chromatograms are presented as Figs. 8 and 9.

4.4.3: GC Analysis

Gas Chromatography revealed Palmitic acid to be the most abundant fatty acid in case of crude lipid extract of *A. dussumieri* followed, in decreasing order, by Oleic acid, Stearic acid, Palmitolic acid, Myristic acid and Linoleic acid. In case of *O. militaris* the most abundant component was Palmitic acid, followed in decreasing order by Oleic acid, Palmitolic acid, Stearic acid, Myristic acid, Lauric acid and Linoleic acid (Figs. 10 and 11).

4.5: Discussion

The results indicate the large number of proteins associated with the catfish mucus. Al-Hassan *et al.* (1982) had shown that the bulk of the epidermal secretion of the Arabian Gulf Catfish is a complex mixture of proteins, and that the soluble protein fraction from the epidermal secretion was

a complex mixture with at least 14 electrophoretically distinguishable components. The soluble fraction from the epidermal secretion of both the species in the current study, also revealed this to be containing at least 16 electrophoretically distinguishable proteins.

Cameron and Endean (1973) indicated, based on the types of secretory cells, that the epidermal toxins of fishes are proteinaceous in nature, although non-proteinaceous materials are also certainly involved in marine toxins (Russel and Brodies, 1974). The proteinaceous nature of the toxin in the epidermal secretions of various catfishes and fishes from other families has been established by a number of workers (Primor and Zlotkin, 1975; Shiomi *et al.* 1987, 1988, 1990; Ali *et al.* 1989).

Al-Hassan *et al.* (1982) also reported that the souble protein fraction contained a wide variety of enzymic activities. The presence of a cytochrome C-like material in the mucus of both the species currently studied indicate such an enzymic activity.

The fatty acids that were discernible had a larger contribution of C-18 (Stearic, Oleic and Linoleic acids) followed by C-16 (Palmitic and Palmitoleic acids), C-14 (Myristic acid) and C-12 (Lauric acid). Goldberg *et al.* (1988) have shown the toxic skin secretions of trunkfishes (Family Ostraciidae) contained β-substituted choline chloride esters of palmitic acid. The presence of linoleic acid also assumes significance since it is converted into arachidonic acid, which is a precursor to prostaglandins.

Fig. 8

Showing chromatogram of combined lethal factors of *A. dussumieri*

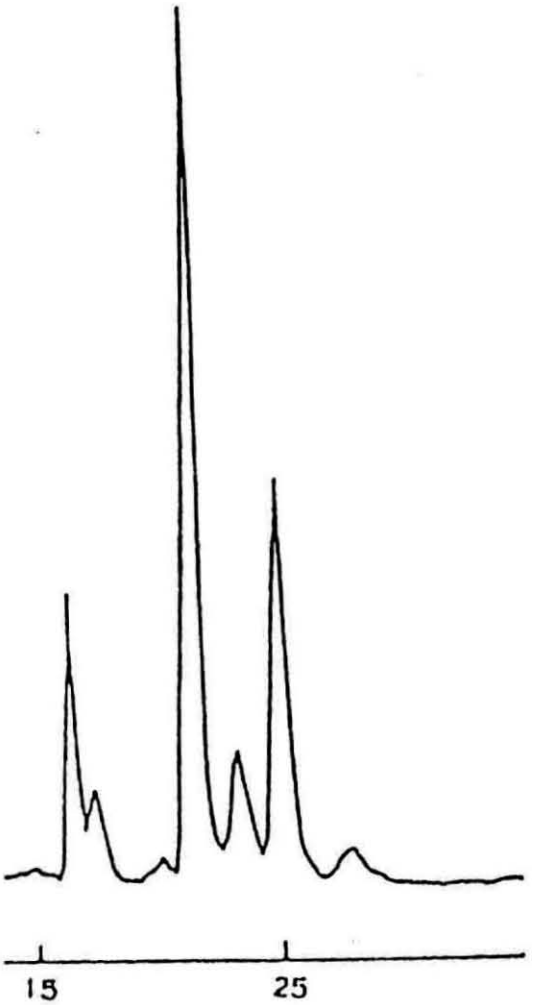

15
25
RT(MINUTES)

Fig. 9

Showing chromatogram of combined lethal factors of *O. militaris.*

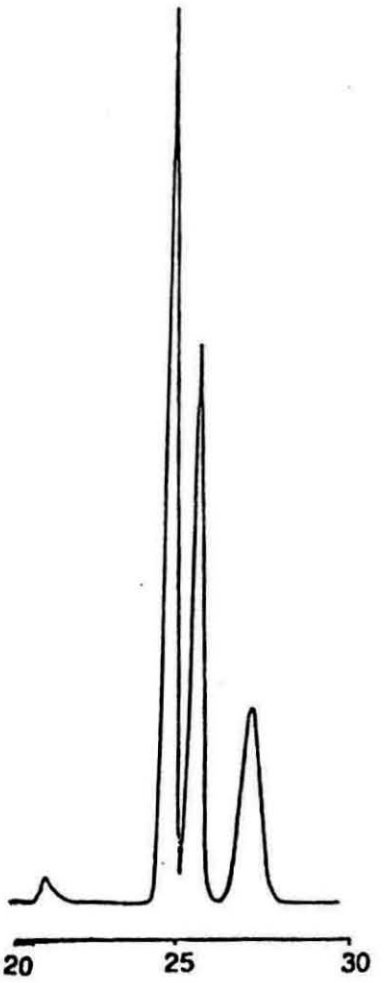

20
25
30
RT(MINUTES)

Fig. 10

Chromatogram showing fatty acid profile of *A. dussumieri*

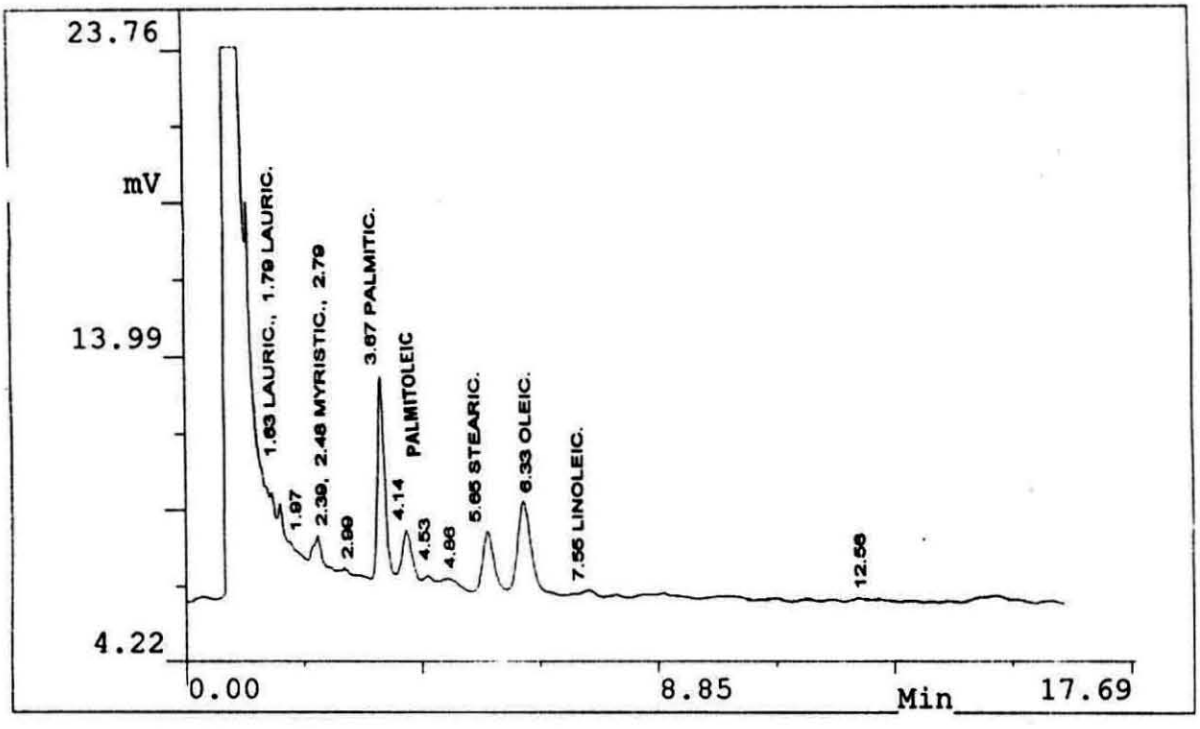

23.76
mV
13.99
4.22
1.63 LAURIC., 1.79 LAURIC.
1.97
2.39, 2.48 MYRISTIC., 2.79
2.89
3.67 PALMITIC.
4.14
PALMITOLEIC
4.53
4.86
5.65 STEARIC.
6.33 OLEIC.
7.55 LINOLEIC.
12.56
0.00
8.85
Min
17.69

Fig. 11

Chromatogram showing fatty acid profile of *O. militaris*.

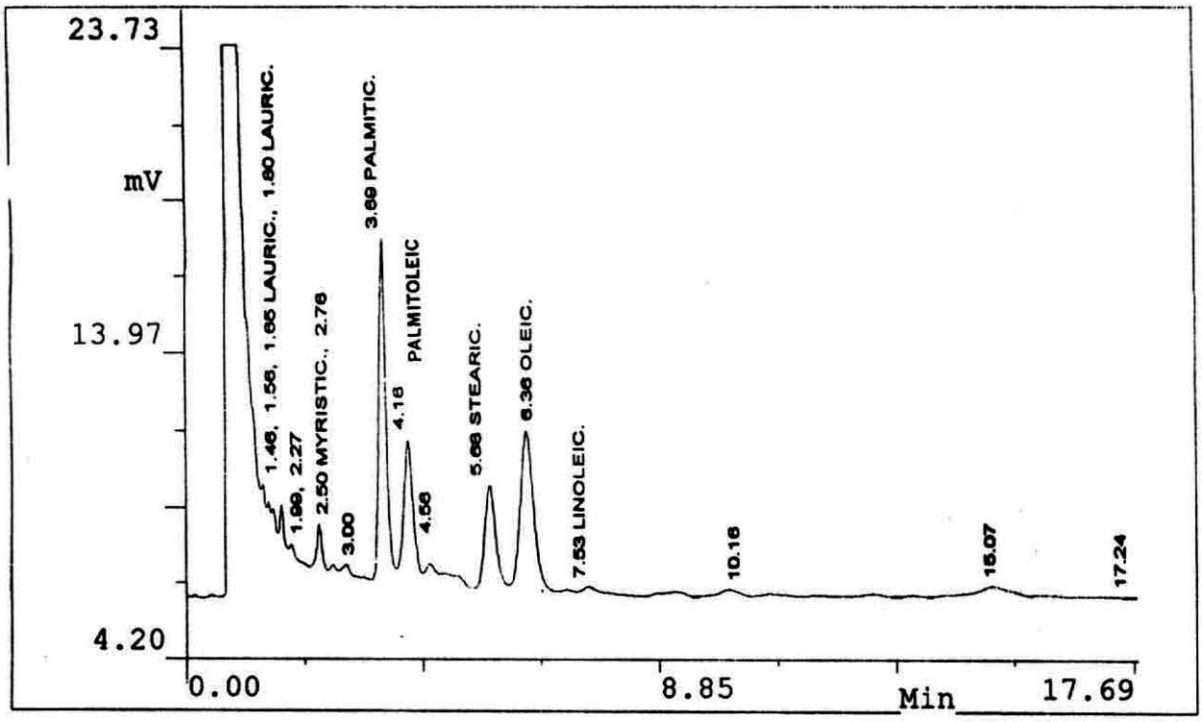
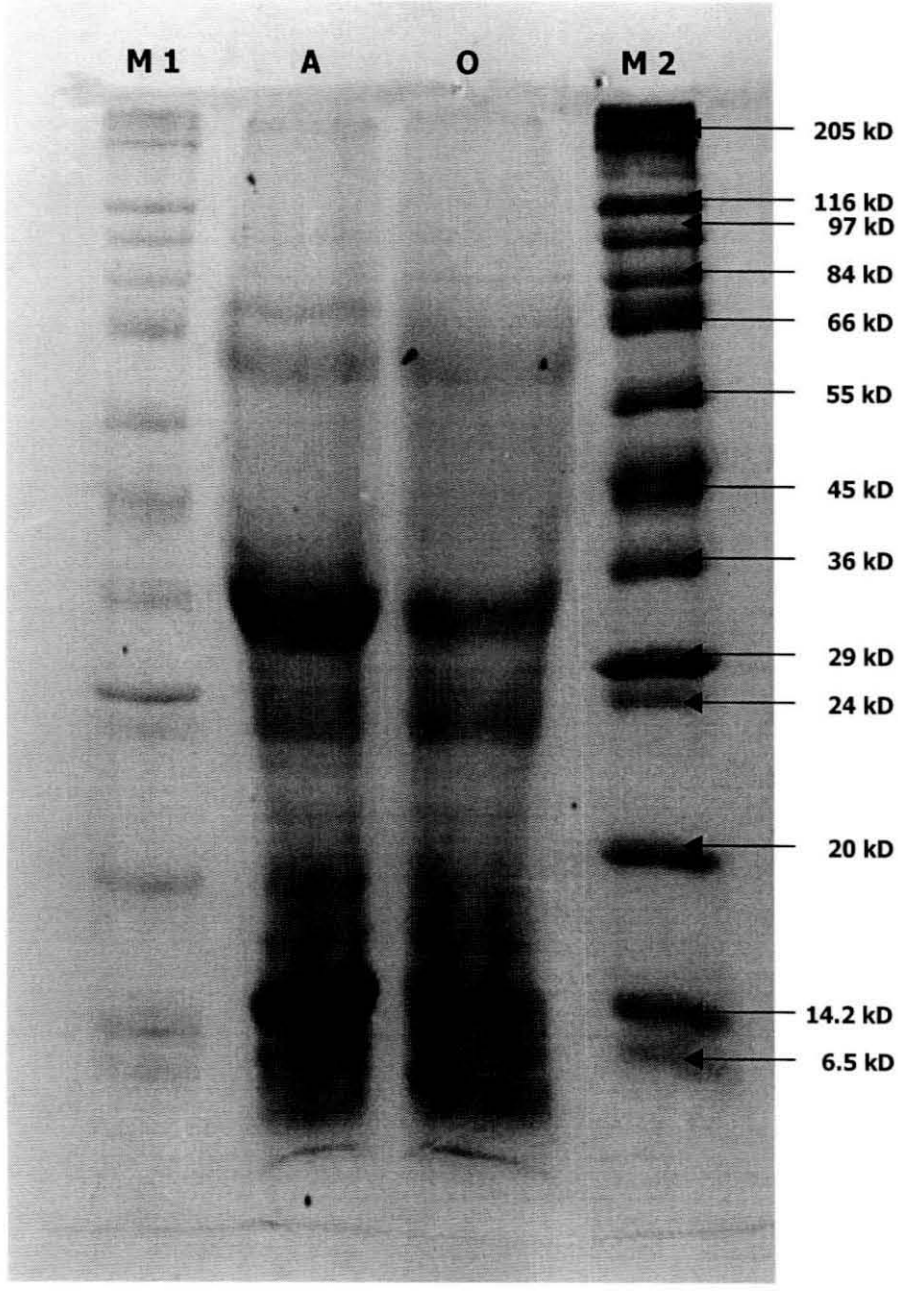

23.73
mV
13.97
4.20
1.46, 1.58, 1.65 LAURIC., 1.80 LAURIC.
1.88, 2.27
2.50 MYRISTIC., 2.76
3.00
3.69 PALMITIC.
4.16
PALMITOLEIC
4.56
5.68 STEARIC.
6.36 OLEIC.
7.53 LINOLEIC.
10.16
15.07
17.24
0.00
8.85
Min
17.69

Plate 15

Showing SDS–PAGE of crude mucus toxin of *A. dussumieri* and *O. militaris*

Lane A: Crude mucus sample of *A. dussumieri*
Lane O: Crude mucus sample of *O. militaris*
Lanes M1 and M2: Wide Range Molecular weight marker (Sigma M4038)

Protein	Mol.Wt. (kD)
Myosin, Rabbit Muscle	205
β-Galactosidase, *E. coli*	116
Phosphorylase b, Rabbit Muscle	97
Fructose-6-phosphate Kinase, Rabbit Muscle	84
Albumin, Bovine Serum	66
Glutamic Dehydrogenase, Bovine Liver	55
Ovalbumin, Chicken Egg	45
Glyceraldehyde-3-phosphate Dehydrogenase, Rabbit Muscle	36
Carbonic Anhydrase, Bovine Erythrocytes	29
Trypsinogen, Bovine Pancreas	24
Trypsin Inhibitor, Soybean	20
α-Lactalbumin, Bovine Milk	14.2
Aprotinin, Bovine Lung	6.5


M 1
A
O
M 2
205 kD
116 kD
97 kD
84 kD
66 kD
55 kD
45 kD
36 kD
29 kD
24 kD
20 kD
14.2 kD
6.5 kD

CHAPTER 5 . WOUND HEALING

5.1: Introduction

There is a tremendous level of worldwide interest in marine natural products with therapeutic potential in industry, academia and government research laboratories, largely because natural products generally continue to be viewed as one of the few *de novo* sources of drug discovery, yielding unorthodox and often unexpected chemical structures that offer novel points of departure for molecular modification leading to clinically available drugs (Mc Conell *et al.* 1994). During the years from 1986 to 1991, the total number of novel compounds increased by 20%; anti-infectives were reduced by 24% and pharmacologically active compounds increased by 87% (Horan, 1994)

The major work of marine toxinologists, for the past decade, has been the search of potential pharmaceuticals. It is virtually difficult to single out a particular bioactive molecule that will find a place in medicine. However, many compounds have shown promise. The art by which marine organisms elaborate bioactive molecules is fascinating. Marine environment provides different biosynthetic conditions to organisms that live in it. Marine organisms generally live in symbiotic association. The pathway of transfer of nutrients between symbiotic partners is of great importance and raises questions about the real origin of the metabolites produced by the association.

The interest in marine products is not new in the history of mankind. Way back in the ancient period of the Phoenicians in about 600 BC, marine molluscs were being used to extract royal purple dye and it was considered to be the most flourishing industry during that period. The royal purple dye was

considered to be a fortune at that time, which, according to some historians, was worth 10-20 times its weight in gold. As many as 10,000 marine molluscs were required to extract 1 gm of the dye. During the past three decades, there had been an increse in the awareness of the remarkable potential that marine flora and fauna have in areas as diverse as health, food additives, materials for orthopedics, thermostable polymerases for polymerase chain reaction and bioadhesive materials.

The fields of marine terpenoids, steroids, saponins, glycolipids, polysaccharides, phenolics, amino acids, alkaloids, peptides, nucleosides and prostanoids are firmly established. Didemin B, one of the several didemnins isolated from the tunicate *Tridemnum*, is selected for clinical trials as an anti-tumor agent (Bhakuni, 1998). Some of the most important bioactive compounds that are under physiological and pharmacological evaluation include peptides as Na^+ blockers or as an exciting agent for muscle and nerves, marine purine arabinosides for their antiviral activity, anti parasitic agents, etc. (Bhakuni, 1997; Mallik, 1997).

Wound healing is a complex phenomenon accomplished by several processes. Compounds from terrestrial sources, more specifically from plants have been in vogue for a long time as wound healers, both in India and abroad. In the modern pharmacology also such plants occupy a significant position. However, wound healing activity in marine organisms have not been given due importance, though scattered information is available on this aspect.

In view of reported wound healing activity in the epidermal gel secretion of Arabian Gulf catfish, *Arius thalassinus*, it is tempting to systematically evaluate the healing potential of epidermal secretion from the

The basic principles of the optimal wound healing are minimizing tissue damage, debriding nonviable tissue, maximizing tissue perfusion and oxygenation, proper nutrition and moist wound healing environment (Pierce and Mustoe, 1995). Until recently, no pharmacologic agents that could reproducibly accelerate wound healing had been identified (Grotendorst *et al.* 1985; Mustoe *et al.* 1987). Growth factors are considered candidate therapeutics because they are i) synthesized by, and ii) stimulate, cells required for tissue repair, and they are deficient in chronic wounds (Pierce and Mustoe, 1995). In another observation, they (Pierce and Mustoe, 1995) suggested the role of platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF- β) in tissue repair *in vivo*. Prathiba and Gupta (2000) reported the role of proteoglycans (PGS), a component of extracellular matrix, in modulating the structure and regulating the functions of the skin. They also indicated the possible role of PGS in different phases of wound healing. Buffoni *et al.* (1993) suggested some biochemical parameters, hydroxyproline, protein, deoxyribonucleic acid (DNA) and semicarbazide - sensitive amine oxidase (SSAO) responsible for wound healing, based on their experiments on guinea pigs.

5.2.2: Fish and Wound Healing

As it was stated earlier, till recently there was no pharmacologic agent identified which can accelerate wound healing. The efforts are on to find out an effective wound healer, and for this extracts/secretions of different plants and animal are being screened. Fish, a significant contributor to the animal kingdom, is no more left behind and the skin extract of an Arabian Gulf catfish,

A. thalassinus was found to accelerate healing of wounds by 60%, as reported by Al-Hassan *et al.* (1983) in mice, rats, guineapigs and even in human beings. They emphasized that the edema and swelling of the wounded area subsided and disappeared in a few minutes. However, they did not find any antibacterial activity in the skin secretion.

Presence of vasoactive substance in skin mucus of catfish, concomitant with prostaglandin release and constriction could play a major role in thrombosis, inflammation and stimulation of cell proliferation (Al-Hassan *et al.* 1985). In another observation (Al-Hassan *et al.* 1986*a*) found clinical and pharmacological properties like accelerated clotting, vasoconstriction, hemolysis, hemagglutination of RBC, protease and nuclear activity to be present in the skin secretion of the Arabian Gulf catfish.

Mat Jais *et al.* (1999) reported antinociceptive activity of *Channa striatus* extracts in mice. They studied the antinociceptive effects of whole fillet and mucus extract from the *C. straiatus*. In the abdominal constriction test, both fillet and mucus extract exhibited concentration-dependent inhibition of the writhing response. Both the extracts had the property to enhance the antinociceptive activity of morphine. Further, Dambisya *et al.* (1999) suggested that the active ingredients of these extracts were relatively stable over a wide range of temperatures and between pH 6.0 and 8.0.

Lipid extracts of epidermal gel secretions have been shown to contain platelet activating factor activity at levels more than 5,000 times the threshold levels required for normal platelet activation that is required for the wound healing process (Al-Hassan *et al.* 1987). They also observed accelerated cell

(polymorphonuclear leucocytes, endothelial cells, neutrophills and fibroblasts) migration rates into wound site, which was essential for fast wound healing.

Al-Hassan *et al.* (1990) devised a novel and simple method of injecting an aqueous extract from the skin of Arabian Gulf catfish, *A. bilineatus*, to treat diabetic foot ulcers in a diabetes mellitus patient without side effects or atrophy. The treatment resulted in natural debridation of necrotic tissues, invasion with angiogenesis and granulation tissues, and also in regaining sensation. The rate of healing was proportional to the amount of healing materials.

5.3: Material and Methods

Assessment of wound healing activity of the mucus of the two marine catfishes, *A. dussumieri* and *O. militaris* was carried out at the Pharamacology Division of Central Drug Research Institute, Lucknow. The lyophilised mucus extract was taken under dry ice from C.I.F.E., Mumbai and stored at -20^0 C. Two models for wound healing activity were tried in the present study.

5.3.1: *Ex ovo* Model

The Chick Chorioallantoic Model (CAM) was used as the *ex ovo* model. In this method nine-day-old fertilized chick eggs were selected and a small window of 1.0 cm^2 made in the shell. A small hole was drilled at the air space and air was sucked to bring the membrane down (Lobb *et al.* 1985).

The window was opened and a sterile disc of methyl cellulose containing different concentration 40 µg and 80 µg of lyophilized mucus extract was placed inside the egg at the junction of two blood vessels. The window

was resealed by tape and the eggs were incubated at 37^0 C in a BOD incubator for 72 hours. The windows were then opened and vessel formation was observed in terms of numbers as also the thickness of the vessel, and finally compared with the eggs containing disks without any lyophilized mucus extract. A positive control was also maintained in the same way.

5.3.2: *In vivo* Models

Albino guinea pigs of either sex (300-350 g) obtained from the CDRI animal-breeding colony were used in the experiment. The guinea pigs were anesthetized with ether and the hair of the lateral sides of the body was clipped off. Using sterile techniques four circular wounds two on each sides, of full thickness, completely transdermal of 8 mm diameter were made with the help of a biospy punch (Acuderm, Louderole, USA). The animals were allowed to recover and housed individually in metallic cages kept under standard animal house conditions. The animals were maintained on food and water *ad libitum.*

Lyophilized mucus extracts of *A. dussumieri* and *O. militaris* was mixed with propylene glycol in the concentration of 0.1%, 0.25%, 0.5% and 1% and applied twice daily for seven days on the wound @ 20 µl/wound. The control group received an equal volume of propylene glycol. All experiments were carried out in triplicate sets.

On the eighth day, the animals were sacrificed and subjected to three types of assessment of wound healing: i) the percentage change in the wound contraction by measuring the area of the wound that was still open; ii) collagen formation in the newly formed tissue using hydroxy proline as the marker; and

iii) lysyl oxidase content in the newly formed tissue, as an indicator of the cross linking of collagen.

i) Area of Wound: The surface area of healing wounds were measured by tracing the boundary of still - open wound on the 8th day on semi-transparent paper and the area was calculated using a graph paper.

ii) Hydroxy Proline Assay: This was performed following the method of Woessner (1961) using reagents, the preparation of which are given under Annexure5. From each animal two wounds were pooled for hydroxyproline estimation. Tissues of two wounds were kept at 110^{0} C for one hour and at 60^{0} C for four hours in an oven. Dried tissues were taken out and placed in a glass test tube, containing 5 ml of 6N HCL. The test tubes were sealed and hydrolyzed for four hours at 130^{0} C. The tubes were then opened and 5 ml of 6N NaOH was added to neutralize the medium. A few drops of methyl red indicator were added to ascertain the final pH.

Standard of hydrodxy proline was prepared containing 0-5 µg of hydroxy proline in 2 ml of distilled water.

To each test tube 1 ml of chloramine T was added, and shaken well and allowed to stand for 20 minutes at room temperature. Then 1 ml of perchloric acid was added to each tube in the same manner and the test tubes were kept at room temperature for 5 minutes. Finally 1 ml of p-dimethylamino benzaldehyde solution was added and the tubes were placed in a water bath at 60^{0}C for 20 minutes, and then cooled under tap water for 5 minutes. The developed color is stable for 1 hour, and within this time the absorbence of the solution was determined spectrophotometrically at 557 nm.

iii) Lysyl Oxidase Assay: The enzyme was assayed fluorometrically following the method of Chitra *et al.* (1998 a) using reagents, the preparation of which are given under Annexure 6. Tissue samples were homogenized in 0.02M potassium phosphate buffer, pH 8.2, containing 1.5M urea. Each sample was then sonicated, 10 bursts, each of 5 seconds and finally centrifuged at 4^0C for 15 minutes at 4,000 rpm. The supernatant obtained was assayed for lysyl oxidase enzyme.

The assay mixture contained 250 µg sodium homovanillate, 40 µg horse radish peroxidase, 100 µM potassium phosphate buffer of pH 8.2 and 200 µl 0.01 M butyl amine substrate in a final volume of 3 ml. The reaction was started by the addition of 0.1 ml of lysyl oxidase. Controls that were containing β-aminopropionitrate (BAPN), and were lysyl oxidase-free and substrate-free, were run simultaneously. The tubes were incubated at 37^0 C for 30 minutes and the reaction stopped by placing the tubes in ice. Fluorescence measurements were made at an emission wavelength of 420 nm with excitation at 320 nm. Lysyl oxidase activity was expressed as arbitrary spectroflurometric units/ mg protein.

5.4: Results

5.4.1: *Ex ovo* Model

The results from the Chick Chorioallantoic Model (CAM) studies are presented in Table 18 and Plates 16 and 17. The mucus extract of both the fish had shown angiogenic activity *viz.* increase in both the number and thickness of blood vessels, as compared to the control at a dose of 40 µg. But at a dose

of 80 µg, mucus extract of *A. dussumieri* exhibited only moderate activity, whereas that of *O. militaris* exhibited marked activity.

5.4.2: *In vivo* Models

i) Area of Wound: The results are presented in Tables 19 and 20 and Figs. 12 and 13. The surface area of healing wound after seven days had shown different degrees of contraction with different doses. In case of *A. dussumieri*, a 0.1% extract was found to effect the highest wound contraction with 22.5% change in wound area as compared to the control, and a 1% dose caused the least wound contraction of 2.15%. The dose of 0.25% and 0.50% were found to have 8.25% and 7.00% change in wound area as compared to the control.

The mucus extract of *O. militaris* at doses of 0.25% and 0.50% were found to have the highest wound contraction activity of 30% and 14.25% respectively, when compared to the control while 0.1% and 1% doses of the extract were least effective.

ii) Hydroxy proline Assay: The results are presented in Tables 21 and 22. Wounds treated with 0.25% and 0.50% mucus extract of *A. dussumieri*, on the 8th day, had the highest level of hydroxy proline of 24.93 and 14.19 mg/gm tissue respectively, as against the control. Wounds treated with the same dose of crude mucus extract of *O. militaris* were also found to have the highest hydroxy proline level of 25.52 and 16.14 mg/gm tissue on the 8th day.

In both the cases, doses of 0.1% and 1.00% had caused very little changes in the level of hydroxy proline as compared to the control.

iii) Lysyl oxidase Assay: The results are presented in Tables 21 and 22. Lysyl oxidase enzyme levels exhibited an increasing trend with increase in dose i.e., the lysyl oxidase was found to be dose dependent. At doses of 0.1%, 0.25%, 0.50% and 1.00%, lysyl oxidase levels were recorded at 8.57, 9.09, 10.01 and 11.60 SFU/mg protein respectively in the case of *A. dussumeiri*, and for the same doses in case *O. militaris* at 8.62, 9.17, 9.46 and 10.17 SFU/mg protein.

5.5: Discussion

Wound healing is a process that is fundamentally a connective tissue response. Initial stage of this process involves an acute inflammatory phase followed by a synthesis of collagen and other extracellular macromolecules which are later remodeled to form a scar (Chithra *et al.* 1998 b); extensive turnover (degradation and biosynthesis) of the connective tissue is evident, requiring the action of proteolytic enzymes like collagenase and cathepsins.

Angiogenesis, the growth of new capillary blood vessels, is important in normal processes such as development of the embryo, formation of a corpus luteum and wound healing. It is also a component in pathological processes such as chronic inflammation, certain immune responses and neoplastic (Taylor and Folkman, 1982).

Topical application of the crude mucus extract of both the catfishes in the present study exhibited angiogenesis and also increased both collagen and degradation in the matrix of a healing wound. This enhanced turnover is reflected in the levels of excreted hydroxyproline. There was an increase in the levels of lysil oxidase, which may lead to higher crosslinking of the newly

formed collagen. Similar results were shown in the case of *Aloe vera*, a medicinal plant belonging to family Liliaceae (Chitra *et al.* 1998 *a* & *b*).

The observed results clearly indicate a potent wound healing activity in the crude mucus extract of both the species. Similar wound healing property has been reported for the mucus of the Gulf catfish *A. thalassinus* by Al-Hassan *et al.* (1983). Shukla *et al.* (1999) studied the wound healing property of asiaticoside isolated from *Centella asiatica* using a model similar to the present one; in guinea pig punch wounds, topical application of 0.2% solution of asiaticoside of 0.2% solution of asiaticoside produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and better epithelialization. Considering the fact that their sample was a purified component as against a crude extract in the present study, the observed value of around 25% increase in hydroxyproline content upon topical application of 0.25% mucus extract is noteworthy. Lysyl oxidase levels increased with increasing doses of application but the range had been very narrow. Thus enhancement of cross-linking of collagen by the topical application of catfish mucus can be considered only marginal.

Wound area studies indicate a faster wound healing upon topical application of the crude extract but the activity was maximum at the lowest dose applied *viz.*, 0.1% in case of hydroxyproline assay also, the second lowest dose of 0.25% only elicited the highest level of hydroxyproline increase. It can, therefore, be deduced that at higher doses the wound healing activity may not be pronounced or effective.

Table 18. Showing angiogenesis in 12 days old chick egg after treatment with *A. dussumieri* and *O. militaris* mucus extracts

Name of the fish	Dose of 40 µg	Dose of 80 µg
A. dussumieri	+	++
O. militaris	+	+++

+ : Slight

++ : Moderate

+++: Marked

Table 19. Showing the change in wound area (mm^2) of guinea pig after topical treatment with different doses of *A. dussumieri* mucus extract

Dose (%)	Wound area (mm^2)			Percentage change in wound area as compared to control
	0 Day	7^{th} Day	Decrease	
0.1	52.0	35.0	17.0	22.5
0.25	51.0	41.5	9.5	8.25
0.50	53.0	46.0	7.0	7.00
1.00	52.0	50.0	2.0	2.15

Table 20. Showing the change in wound area (mm^2) of guinea pig after topical treatment with different doses of *O. militaris* mucus extract

Dose (%)	Wound area (mm^2)			Percentage change in wound area as compared to control
	0 Day	7th Day	Decrease	
0.10	53.0	51.0	2.0	0.35
0.25	52.0	28.0	24.0	30.00
0.50	52.0	37.0	15.0	14.25
1.00	53.0	49.0	4.0	1.75

Table 21. Showing the increase in hydroxyproline and Lysyloxidase content of the wounds (compared to control) in guinea pig punch model upon topical application of *A. dussumieri* extract

Dose (%)	Hydroxyproline (mg/gm tissue)	Lysyloxidase (SFU/mg protein)
0.1	2.92	8.57
0.25	24.93	9.09
0.50	14.19	10.01
1.00	1.90	11.60

(SFU – Spectro Flurometric Unit)

Table 22. Showing the increase in hydroxyproline and Lysyloxidase content of the wounds (compared to control) in guinea pig punch model upon topical application of *O. militaris* extract

Dose (%)	Hydroxyproline (mg/gm tissue)	Lysyloxidase (SFU/mg protein)
0.1	7.96	8.62
0.25	25.52	9.17
0.50	16.14	9.46
1.00	3.55	10.17

(SFU – Spectro Flurometric Unit)

Fig. 12: Change in area (mm^2) of Guinea pig wounds after topical treatment with different doses of *A. dussumieri* mucus extract

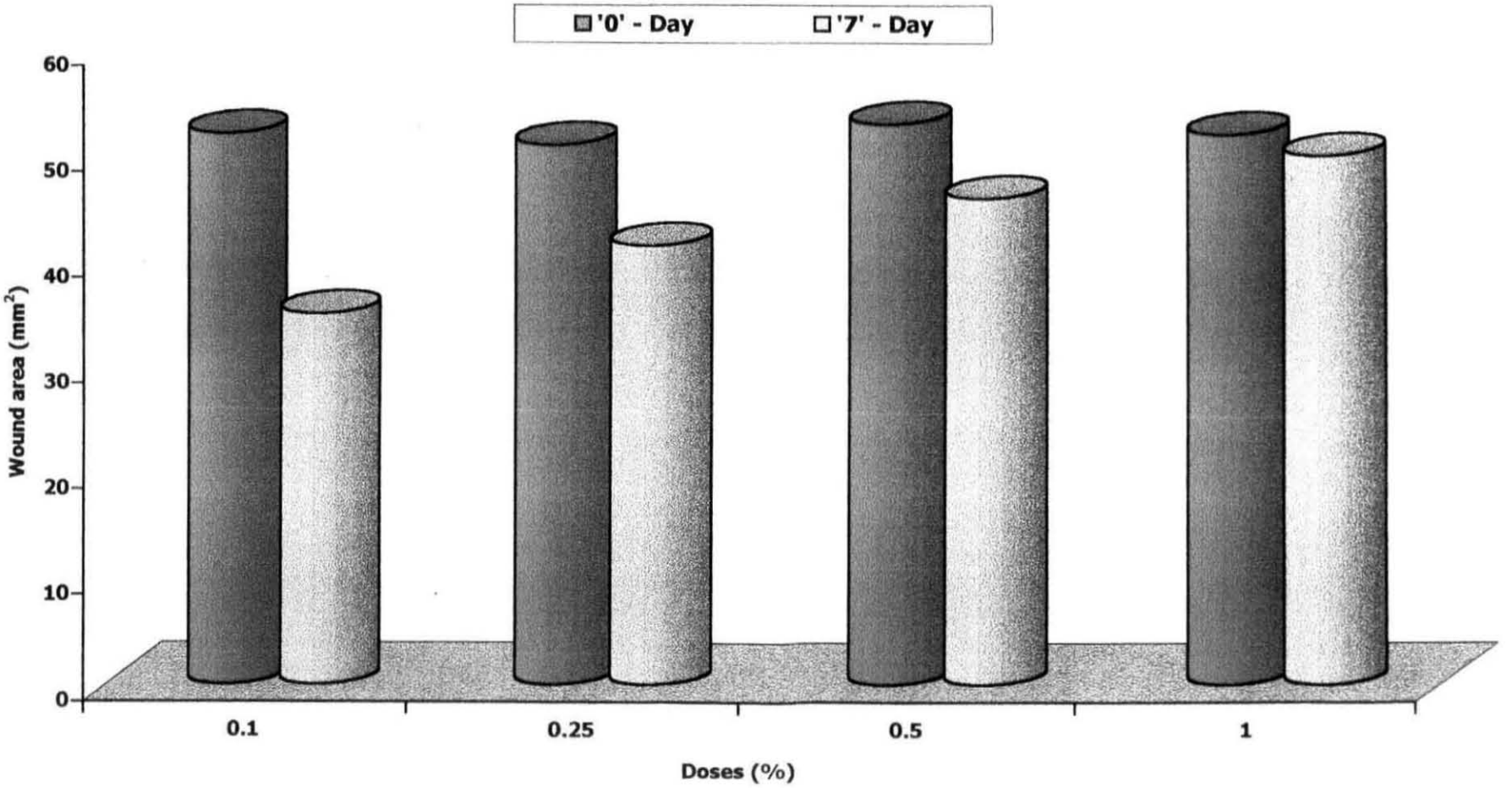


Fig. 13: Change of area (mm^2) of Guinea pig wounds after topical treatment with different doses of *O. militaris* mucus extract

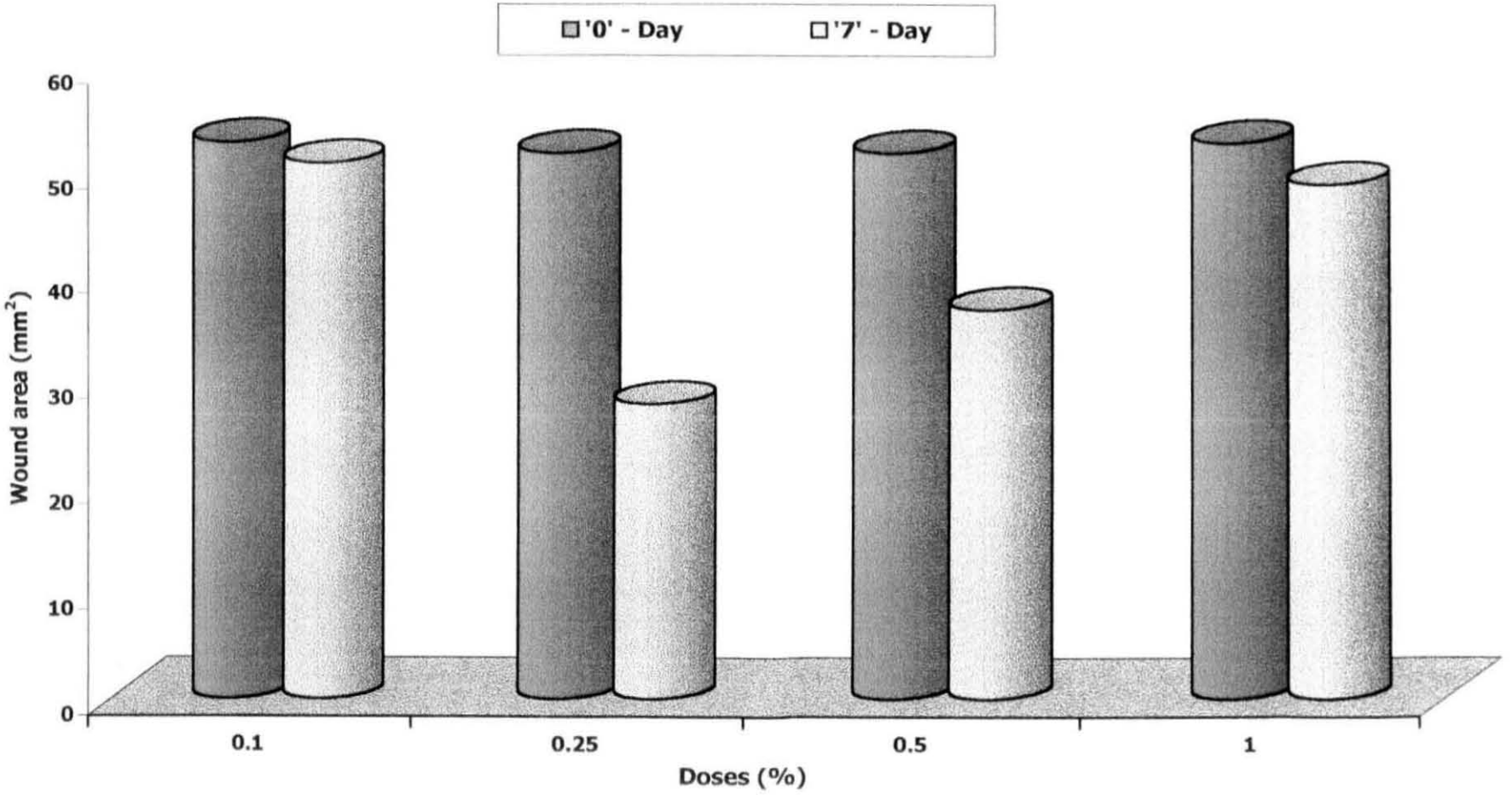


Plate 16

Showing angiogenesis in hen's egg on the 12 th day after treatment with 80 μg of *A. dussumieri* mucus extract

CP: Control (without mucus extract)
6: Positive control
1: Sample (mucus extract)

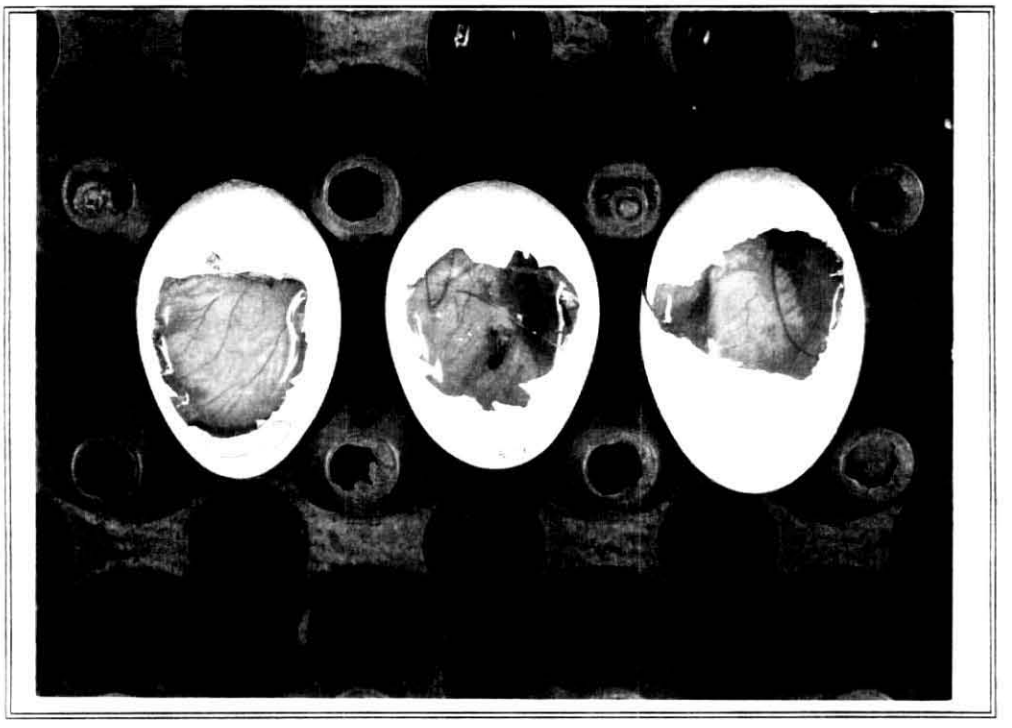
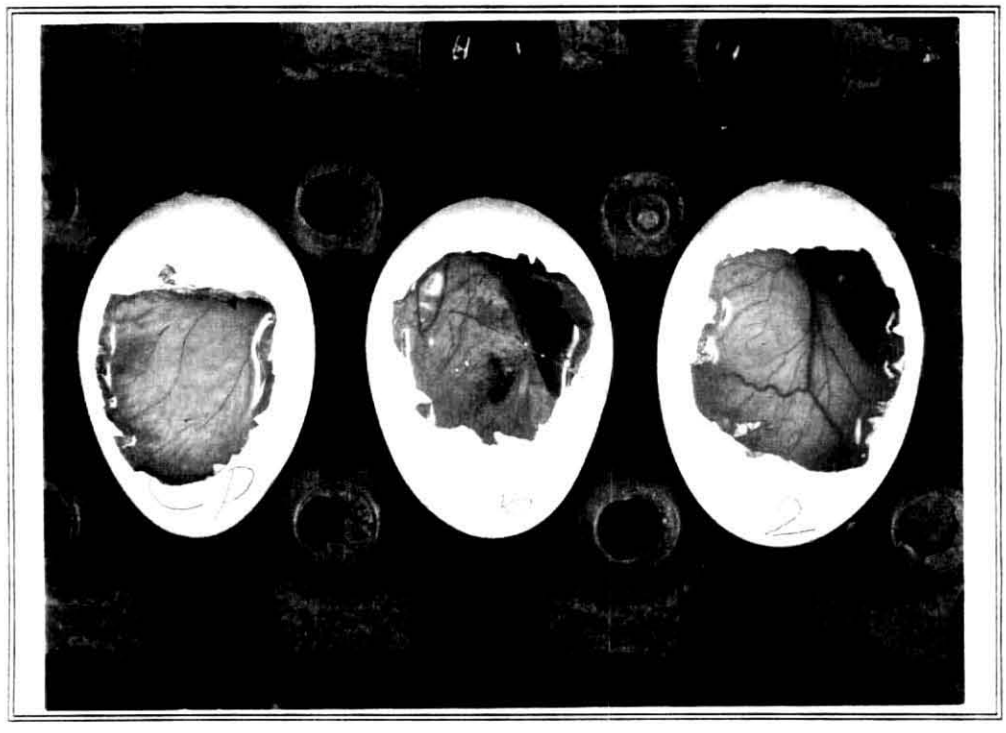


Plate 17

Showing angiogenesis in hen's egg on the 12 th day after treatment with 80 μg of *O. militaris* mucus extract

CP: Control (without mucus extract)
6: Positive control
2: Sample (mucus extract)



CHAPTER 6 : GENERAL DISCUSSION

6.1: Significance of the Crinotoxins in the Two Fishes Studied

Although the functions attributed to fish mucus are many (Shepard, 1994), it is now generally agreed that it has a defensive role especially in scaleless fishes such as catfishes since the mucus may partly fill the protective role of scales (Al-Hassan *et al.* 1986). The epidermal gel secretion of *A. thalassinus* may act in a further protective role (Al-Hassan *et al.* 1985); the lectin-like activity may contribute to a general defensive mechanism which blocks infestation by water contaminants or possibly aids in the repair of damage to the superficial tissues of the catfish. Al-Hassan *et al.* (1985) have also shown that *A. thalassinus* secreted epidermal mucus containing several toxic components in an apparently defensive type response but these components did not appear to function as toxic, repellant or fright substances; Shiomi *et al.* (1987) opined such a condition could be true for *Plotosus lineatus* also. Randall *et al.* (1981), based on their studies involving the Moray eel *Lycodontis nudivomer*, postulated that in eels, the toxins in the epidermal secretion could be a deterrant to external parasites. Reproduction and spawning may be themselves extremely stressful events (Reddy and Leatherland, 1998) associated with depressed plasma immunoglobin titres (Suguki *et al.* 1997), increased disease susceptibility (Pickering and Christiej, 1980), marked lymphocytopemia (Pickering, 1986) and increased prespawning mortality (Mills, 1971). Thus, in scaleless fishes, secretion of a copious amount of mucus can be expected during the stressful event of reproduction, espcially in the spent fishes which are extremely stressed.

Originally thought to be induced by heat shock, the production of stress proteins (or Heat Shock Proteins, HSP) are now shown to produced by environmental stress, pathophysiological state, as also non-stressful conditions such as cell-cycle, growth factors and development and differentiation (Morimoto *et al.* 1990). It is, therefore, possible that mucus of a spent, and thereby stressed, fish might contain stress proteins.

The present investigation had revealed the crinotoxicity of the epidermal secretions of both the species of catfish to be maximum in spent fishes, during November in *A. dussumieri* and September in *O. militaris*, and the lowest Condition Factors were also recorded during the same period. Condition Factor being indicative of general well being of fish (Le Cren, 1951) values less than 1.0 show the fishes were not in good health, or in other words, stressed, and therefore sucessptible to diseases, infestations, etc.

It might, therefore, be possible that these fishes secreted stress proteins that not only are toxins and hemolysins but also are wound healing in nature. Support for this line of hypothesis comes from Shukla *et al.* (1998) who have shown the 68 kD protein in the case of wounded guinea pigs appeared to be a heat shock protein that was overexpressed in normal wounds and synthesis resumed the basal level with repair of wounds. Forsyth *et al.* (1997) also have demonstrated the production of HSP 70 in Coho salmon stressed by a bacterial kidney disease. The observation of electrophoretic bands between 66 and 84 kD molecular weights in the present study could be indicative of HSP 68 or 70 being produced, by an inherent defensive mechanism, by these catfishes under stress.

Healing process involves the *de novo* or increase synthesis of heat shock proteins, growth factors and their receptors; these proteins are site specific and remain as long as wounds healed (Shukla *et al.* 1998). It was also suggested by Shukla *et al.* (1998) that exogenous application of such HSPs would help wound healing. Thus, it is possible that the HSP 68 or 70 that might have been present in the mucus, would have complemented the HSPs either produced *de novo*, or increasingly synthesized by the guinea pig could have accelerated the wound healing as observed during the present study.

Al-Hassan *et al.* (1986) reported that the vasoconstrictor components in the mucus of the Gulf catfish affected bleeding and caused prostaglandin release concomitant with contraction and that these played a major role in thrombosis, inflammation, and cell proliferation all of which were useful in wound healing. Such a prostaglandin release would also cause the production of stress proteins (Santoro *et al.* 1990).

Because of their ubiquitous nature, their increased synthesis after different kinds of stresses (among which heat shock, Chen *et al.* 1987), and their well-known physiological role in the production of fever (Dinarello and Wolff, 1982; Coceani *et al.* 1989), prostaglandins could be considered as possible intracellular signals for induction of heat shock proteins (Santero *et al.* 1990).

Thus the cumulative effects of such a prostaglandin release could have increased the HSPs in the mucus and also aided the wound healing as observed in the present study. The fright or shock induced release of lectins which are hemagglutinins may play some general role in a protective or defensive response of various animals (Al-Hassan *et al.* 1987).

Skin mucus of fishes is known to have many kinds of antipathogenic substances such as antibodies, complements lysins and agglutinins (Ingram, 1980). Suzuki (1985) had shown that the hemolysin and hemagglutinin in skin mucus of the Japanese eel *Anguilla japonia* was not transferred from blood plasma but made within the skin.

Several marine biotoxins are both ichthyocidal and hemolytically active, though there is no compelling evidence to suggest that the two activites are directly linked *in vitro*. Ostracitoxin (pahutoxin), holothurin A, prymnesin, asterotoxin (starfish toxin; Hashimoto and Yasumoto, 1960) and one component of *Gymnodinium breve* toxin (Paster and Abbott, 1969) are hemolytically active, though they differ greatly in specific hemolytic activity.

In a few instances, some of the hemolytic properties have been quantified, as the following examples indicate. Ostracitoxin, either natural or synthetic, was found to be a strong hemolysin (Thomson, 1964; Boylan, 1966) even at 0.1 ppm (citrate buffer) and when tested against amphibian, fish and mammalian erythrocytes. Ostracitoxin also caused strong agglutination reactions at a concentration greater than 50 ppm in fish (eleven species) or with rabbit erythrocytes, though not with mouse or human erythrocytes. In contrast, saponin (Merk) or crude holothurin, though hemolytically active, did not produce agglutination in the erythrocytes tested (Thomson, 1968). Boylan (1966) found that a decrease in chain length by four carbons of the synthetic toxin, choline 3-acetoxyhexadecanoate, produced a marked decrease in hemolytic activity.

The hemolytic activity of prymnesin has also been studied. It may be noted that the hemolytic activity is maximum at a pH of about 5.5 (Padilla,

1970), that the kinetics of hemolysis or rabbit erythrocyte suspension have been measured, and that the variation of hemolytic rat with concentration follows a Michaelis-Menten pattern (Martin and Padilla, 1971). Saxitoxin though not a strong lysine, causes passive hemagglutination.

It may be reasonably assumed tht strong lysins attach to the membrane, remove a specific component, and produce a destructive effect. The action of other substances has a more subtle effect, but, possibly, it is as deleterious in impairing other membrane functions (e.g., antigen – antibody interactions).

Thus, the collective properties of the mucus of the two catfishes observed in the present study such as hemolysis, hemagglutination, and edema formation, together with wound healing might be inferred to have a primary defence function in these fishes especially under stress condition.

Al-Hassan *et al.* (1985) have shown that *A. thalassinus* secreted epidermal mucus containing several toxic components in an apparently defensive type response but these components did not appear to function as toxic, repellant or fright substances; Shiomi *et al.* (1987) opined such a condition could be true for *Plotosus lineatus* also. Thus, in case of the two catfishes currently studied, the lethal nature of the mucus could only be a synergistic activity of these various properties.

6.2: Scope for Further Studies

Wound healing activity of the purified fractions of the crude mucus extract of both the catfishes would lead to the development of extremely useful pharmaceutical compounds. The elucidation of their chemical structure

is another important and interesting line of research leading to the synthesis of these novel proteins.

The separation of the possible stress/heat shock proteins at about the 70 kD electrophoretic bands and elucidating their structure and function would be of considerable research interest in unraveling the role of such proteins in not only crinotoxic, but also poisonous and venomous fishes and other aquatic organisms.

The absence of specialized glandular cells in the skin as also the presence of glycoprotein-like substances and adipose tissue observed for the first time in the lumen of the spine open up exciting avenues for further research on the acanthotoxic nature of the catfishes.

CHAPTER 7 : REFERENCES

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ANNEXURE 1

COMPOSITION OF ALSEVER'S SOLUTION

Sodium chloride:	4.15 g/l
Tri sodium citrate:	8.00 g/l
Glucose:	20.50 g/l
Citric acid:	0.55 g/l
Distilled water:	1000 ml

ANNEURE 2

Composition of media (Nutrient Broth)(pH 7.2 + 0.2)

Composition	(g/l)
Peptone:	5.0
Sodium chloride:	10.0
Yeast extract:	2.0
Beaf extract:	1.0

For Vibrio species extra 0.5% NaCl was added into the medium. The ingredients were dissolved in distilled water and made up to one litre. The solution was poured in test tubed and sterilized in autoclave at 121^0 C for 15 minutes.

ANNEURE 3

Composition of media (Antibiotic assay agar)(pH 7.2 + 0.2)

Composition	(g/l)
Trypton:	15.0
Soy trypton:	5.0
Sodium chloride:	10.0
Agar:	15.0

The above ingredients were dissolved in distilled water, made up to 1 l and sterilized in an autoclave at 121^0 C for 15 minutes.

ANNEURE 4

Composition of PAGE Reagents

1. Stock arcrylamide solution: (30%)

Acrylamide	: 29.1 g
Bis acrylamide	: 0.9 g

The mixture was dissolved in a very small quantity of water, then made up to 100 ml using double distilled water and then filtered using whatman No.1 filter paper and stored in amber coloured bottles in refrigerator.

2. Gel buffers:

a. Separating gel buffer:

1.5 M Tris-HCl (pH 8.8) 18.75 ml of 2M Tris was taken and made upto 25 ml after adjusting the pH to 8.8 by using 2N HCl.

b. Stacking gel buffer:

0.5 M Tris-3.028 gm (pH 6.8) pH was adjusted to 6.8 using 2 M HCl and the final volume was raised up to 50 ml using double distilled water.

3. Electrode buffer:

0.0 SM Tris-HCl	: 6.057 g
0.038 M glycine	: 28.527 g
0.1% SDS	: 1 g
Distilled water	: 1 litre

4. 10% SDS stock solution

5. Polymerizing agent

Ammonium persulfate - 10% (Freshly prepared)

6. TEMED (N, N,N^1,N^1- tetramethylethylenediamine)

Composition of 12% Gel:

Separating Gel	: (10.005 ml)
Distilled water	: 3.4 ml
Separating gel buffer	: 2.5 ml
SDS	: 0.05 ml
Acrylamide solution (30%)	: 4.00 ml
Ammonium persulfate	: 0.05 ml
TEMED	: 0.005 ml
Total monomer	**: 10.005 ml**

Stacking gas	: 5.05 ml
Distilled water	: 3.075 ml
Stacking gel buffer	: 1.25 ml
Acrylamide solution (30%)	: 0.067 ml
Ammonium per sulfate	: 0.025 ml
TEMED	: 0.005 ml
Total stack monomer	**: 5.05 ml**

iii) Sample buffer:

Distilled water	: 4.0 ml
Stacking gel buffer	: 1.0 ml
Glycerol	: 0.8 ml
SDS	: 1.6 ml
β-mercaptoethanol	: 0.4 ml
Bromopheonl blue	: 0.2 ml

ANNEURE 5

Composition of Reagents for Hydroxyproline Assay

A. Hydroxy proline standard

- 25 mg of vaccum - dried L-hydroxy proline in 250 ml of 0.001N HCl.
- Dilute with water to get the concentration of 1-5μg/2ml.

B. Buffer- Citric acid monohydrate - 50 g

Glacial acetic acid - 12 ml

Sodium acetate trihydrate - 120 g

Sodium hydroxide - 34 g

Distilled water - 4 l

C. Chloramine T (0.05 M)

Sodium p-Toluenesulfonchloramide - 1.41g

Distilled water - 20ml

Ethylene glycol mono methyl ether

(methyl cellosolve) - 30ml

Buffer - 50ml

D. Perchloric acid(3.15M)

70% perchloric acid - 27ml

D.W. - 100ml

E. P-Dimethylaminobenzaldehyde (20% solution)

Take 20g of P-dimethylaminobenzaldehyde in a flask and Ethylelen glycol mone methyl ether (methyl cellosolve) to give a final volume of 100ml.

(Freshly prepared solution should be used).

ANNEURE 6

Composition of Reagents for Lysyl oxidase Assay

1. 0.02 M Potassium phosphate buffer (pH 8.2);
2. Urea 1.5M
3. Sodium homovanillate 250μg
4. Horse radish peroxidase 40 μg
5. Butyl amine (0.01M)-200 μl
6. ɨ-amino propionitrate (0.1mm)-100μl (Inhibitor of enzyme).